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SULFONAMIDE-RESISTANT GONORRHEA TREATED WITH UREA AND SULFONAMIDE BY MOUTH

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AS EFFICACIOUS as the sulfonamides have been in the treatment of gonorrhea, the problem of sulfonamide-resistant cases in the Armed Forces, until recently, has been an important one. It constituted as many as 10 to 20 per cent of cases. Penicillin, however, has now made gonorrhea in any of its forms curable in practically all cases.

But before penicillin became available to us, sulfonamide-resistant cases were frequently difficult to treat and resulted in much loss of time from duty. In an attempt to find a more successful method of treatment for these cases than anterior urethral instillations of silver salts and a safer method than fever therapy we were encouraged from the following considerations to try urea by mouth.

After D. D. Woods of London[1] and others had demonstrated that para-aminobenzoic acid, peptone, tissue extracts, etc., may be *inhibitors* of sulfonamide activity, further studies showed that such substances as azochloramide,[2] certain purines,[3] and urea may behave as *anti-inhibitors*. Such a substance as urea may act in some way to prevent the inhibitors from interfering with the sulfonamide action.

With this newer knowledge, several investigators have tried urea combined with a sulfonamide as a local dressing to wounds that had not responded previously to a sulfonamide alone.[4] Tsuchiya and his associates[5, 6, 7] found the combined action of the two substances effective against certain organisms in vitro, particularly staphylococci and *B. coli*. When this idea was applied in vivo to wounds, compound fractures, and infected dermatoses, it met with some

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degree of success.[8, 9] When used in this way externally, urea is thought to increase the solubility of the sulfonamide, to enhance its bacteriostatic action, and to prevent the sulfonamide from caking.

Because of its property to increase sulfonamide solubility,[10] Sobin and his associates gave urea internally in an attempt to reduce the toxic effects of the sulfonamides on the kidneys.[11] They administered urea by mouth to rats and considered it to be of some benefit in preventing the renal precipitation of acetylsulfonamide crystals. No toxic effects were encountered.

Such studies of the combined use of an anti-inhibitor, as urea, and the sulfonamides seem important in attempting to broaden our scope of knowledge of the sulfonamide group of drugs.

MATERIAL AND METHODS

At the time this study was undertaken, sulfonamide therapy for gonorrhea in the Army[12] consisted in the administration of 4.0 Gm. of sulfathiazole or sulfadiazine, given in doses of 1.0 Gm. four times daily for five days. The majority responded to this treatment. If it failed, the same course was repeated, or the patient was hospitalized and given 4.0 Gm. of either drug as an initial dose, followed by 1.0 Gm. every four hours, day and night, for five days. If the discharge still persisted the case was labeled "sulfa-resistant."

Patients were selected for this study who had been sent to the Valley Forge General Hospital as being "sulfa-resistant"; their urethral smears were persistently positive for gonococci. The majority had been given sulfathiazole and had had both the above courses of treatment. Several had had as many as five courses. Treatment was withheld for several days after admission to be certain there was no lag in response to their pre-admission therapy, and to obtain additional data. Besides a careful history and physical examination, such studies included complete blood examination, a second- or third-glass urine specimen for albumin and red blood cells, fasting blood urea nitrogen, and 24-hour urine urea. When all these findings were normal the patient was started on urea in the following mixture:

Urea crystals	325.0 Gm.
Distilled water	450.0 c.c.
Simple syrup q.s. ad	1000.0 c.c.

This mixture was tolerated very well and in the dose of two tablespoons three times a day supplied about 30.0 Gm. of urea daily. This treatment alone was given for three days. On the morning of the third day another fasting blood urea nitrogen determination was made and the collection of a second 24-hour urine for urea content was begun. On the morning of the fourth day urethral smears were examined again to determine whether gonococci were still present. The patient was then begun on a sulfonamide, the same drug and in the same dosage which had failed previously.

The urea and sulfonamide were continued together on the fourth, fifth, and sixth days; then the urea was stopped. If there were no complications as shown by symptoms, blood counts, or urinary findings, the sulfonamide was continued for another three days. The urine was examined daily by the "two-glass test."

If, at the end of treatment, any discharge remained, smears, and in some instances, cultures, were made. The patient was then observed for another three weeks and his progress followed by two-glass tests of the urine and smears. The total treatment as outlined above required nine days, three days on urea alone, three days on urea plus sulfonamide, and three days on sulfonamide alone.

RESULTS OF THERAPY

Forty male patients were treated by this method. Thirty-six were white, four Negro. Thirty-eight were given sulfathiazole, two were given sulfadiazine. All of the patients showed persistence of gonococci after three days on urea alone.

Of the 40 cases, 21, or 52.5 per cent, showed a rather dramatic recovery on the urea and sulfonamide; that is, the urethral discharge ceased abruptly and completely. The two cases treated with sulfadiazine are in this group. We have called these 21 cases successful because there was complete clearing of the two-glass urine specimen, and there was complete absence of symptoms and urethral discharge, with no recurrence over a subsequent three-week period of observation. In the majority of these successful cases the discharge and symptoms usually disappeared on the sixth or seventh day of the treatment, that is, on the third or fourth day that the sulfonamides were administered.

In looking for some causes for failure, the 19 cases that did not respond favorably to the combined treatment were compared in certain respects to the 21 successful cases.

TABLE I

SHOWING RELATIONSHIP BETWEEN TIME INTERVAL FROM APPEARANCE OF DISCHARGE TO INSTITUTION OF ORIGINAL SULFONAMIDE THERAPY, AND THE LATER RESPONSE TO UREA-SULFONAMIDE TREATMENT

NO. OF CASES	DAYS OF URETHRAL DISCHARGE BEFORE ANY THERAPY	THERAPY WITH UREA-SULFONAMIDE	
		SUCCESS	FAILURE
31	Immediate	16	15
2	4	0	2
2	7	2	0
1	11	1	0
1	14	1	0
1	35	0	1
2	60	1	1

There was no apparent relationship between the failure of sulfonamide-urea therapy and the interval between onset of the urethral discharge and institution of the original sulfonamide administration (Table I). Of the total group, 31 had had sulfonamide treatment started immediately upon the appearance of a discharge. Sixteen of these were treated successfully by urea-sulfonamide, 15 failed. Two cases that had gone eleven and fourteen days before any therapy was given were both treated successfully with urea-sulfonamide, but 2 cases that had gone only four days, 1 that had gone thirty-five days, and 1 of 2 cases that had gone eight weeks without treatment were failures.

As shown in Table II, the number of previous attacks of gonorrhea seemed to make no difference in the end results of urea-sulfonamide therapy. Five of the total group had had one previous attack of gonorrhea, 3 had had 2 attacks. It might be thought that previous attacks of the disease would build

TABLE II

THE SUCCESS OR FAILURE OF UREA-SULFONAMIDE THERAPY IN TERMS OF PREVIOUS ATTACKS OF GONORRHEA

NO. OF CASES	PREVIOUS ATTACKS	SUCCESS	FAILURE
32	0	18	14
5	1	2	3
3	2	1	2

TABLE III

RESULTS OF UREA-SULFONAMIDE THERAPY AS RELATED TO PREVIOUS COURSES OF SULFONAMIDES

NO. OF CASES	PREVIOUS COURSES	SUCCESS	FAILURE
15	1	9	6
14	2	8	6
6	3	2	4
5	over 3	2	3

some immunity and would make our treatment more successful, or that previous attacks, with chronicity, might promote drug resistance. Neither of these ideas was confirmed.

Since several courses of sulfonamides with failure may indicate a resistant strain, and since resistance of an organism may be acquired by repeated administrations of the sulfonamide drugs, it was thought that perhaps the number of previous courses of sulfonamide therapy may have influenced our negative results. As shown in Table III, the number of previous courses of sulfonamides made no difference in the results with urea-sulfonamides.

On the assumption that for some of these patients the doses of sulfonamides that had been used were not large enough, and that in fact they were not "sulfonamide-resistant" if adequate treatment for them meant larger doses, we carried out the following control study. Fifteen patients of the same type studied with urea-sulfonamides were given double the routine doses of sulfonamide treatment alone. This consisted of an initial dose of 4.0 Gm. of sulfathiazole followed by 2.0 Gm. every four hours, day and night, for five days. Only 3 or 20 per cent, responded to this regime. This method of giving double the dose in similar cases has been used by Wright with recovery in 70 per cent.[13] Our results by this method did not show such a satisfactory response. Four of the failures on larger doses of sulfathiazole alone were then placed on the small regular doses of sulfathiazole and urea; two of them cleared up promptly.

Two patients who had not responded to the regular urea-sulfonamide regime were placed on the larger doses of sulfathiazole and urea. One of them was successful, the other was a failure.

In the way of further control studies, certain sulfonamide-resistant cases were given urea or alkali alone. Five patients were given the 30.0 Gm. of urea alone for 7 days and none of them had any diminution of discharge, or disappearance of organisms from the urethral smear. They all responded when given sulfathiazole in addition to urea, 4 with the usual doses, 1 with the larger doses.

Since many of our patients were given 1.0 to 2.0 Gm. of sodium bicarbonate with each dose of the sulfonamide to prevent renal toxic effects, it was necessary

to rule out any possible benefit from the bicarbonate. As shown by Fox and Rose[14] and Schmelkes,[15] alkalies may enhance the bacteriostatic action of the sulfonamide. Four patients were given sodium bicarbonate alone, 4 others received it with regular doses of sulfathiazole, and in none was there an effect on the gonorrhea. All 12 patients who had failed on the larger (double) dose schedule of sulfathiazole had been given 2.0 Gm. of sodium bicarbonate every four hours. From this we concluded that the alkali we had administered was no factor in the successful results obtained with urea-sulfonamide treatment.

Of the 19 failures in the series, 8 responded to anterior urethral instillations of silver salts. In this small group we gained the distinct impression that these patients responded much more quickly to the silver salts after having had the urea-sulfonamide treatment, i.e., in four or five days, than had previously similar cases which had been treated with sulfonamides alone. Two patients were given fever therapy with success. At about this time small amounts of penicillin became available to us and the other 9 patients responded dramatically to its administration.

Only 5, or 12.5 per cent, of the 40 patients developed any toxic effects from the sulfonamides. In 4 instances these were mild, including headache, drowsiness, conjunctivitis, and microscopic hematuria. One patient had a severe reaction with fever, malaise, and headache after two days of drug treatment. These patients had all received sulfathiazole. There were no untoward effects from the urea.

LABORATORY STUDIES

Before beginning the urea-sulfonamide therapy, the fasting blood urea nitrogen ranged between 14 and 18 mg. per 100 c.c. The urine urea content averaged 25 to 35 Gm. per 24 hours. After the patient had taken urea for three days the fasting blood urea nitrogen ranged between 16 and 20 mg. per 100 c.c. and the 24-hour urine contained between 30 and 40 Gm. of urea.

In our laboratory, when patients are treated with sulfathiazole with the army dosage as outlined, the blood level of drug averages between 4 and 7 mg. per 100 c.c. With the urea-sulfonamide treatment, using the same dose of sulfathiazole, the blood levels of drug ranged between 4 and 8 mg. per 100 c.c.

In 14 of the 21 successfully treated cases cultures of urethral strippings were made at weekly intervals for three weeks. All were negative. In any of the failures in which cultures were made, gonococci persisted.

Laboratory confirmation of this clinical experiment was sought. Using the technique of Goodale and his associates,[16] cultures of gonococci from these resistant cases were made on chocolate agar. In addition to a control, chocolate agar plates were used containing 0.1 mg., 0.25 mg., and 0.50 mg. per cent sulfathiazole. Other chocolate agar plates containing 0.5 per cent, 1.0 per cent, and 2.0 per cent urea were implanted with the same strain of organism. A third set of chocolate agar plates containing both chemicals in combinations of the above strength were also implanted. The results are shown in Figs. 1 and 2.

In Fig. 1 it will be noted that gonococci grew out in significant numbers in all strengths of sulfathiazole alone and urea alone. In these cultures we

did not observe the urea-inhibiting effect described by Kirby.[17] Kirby has reported that 2.0 per cent urea, and often as little as 0.2 per cent strength, definitely inhibits the growth of cultures of B. coli. In all our cultures the gonococci grew readily on the agar-urea plates, occasionally even better than on the control plate. From the 5 patients treated with urea alone for a week, and the lack of any effect of three days of urea in our 40 patients, we feel that

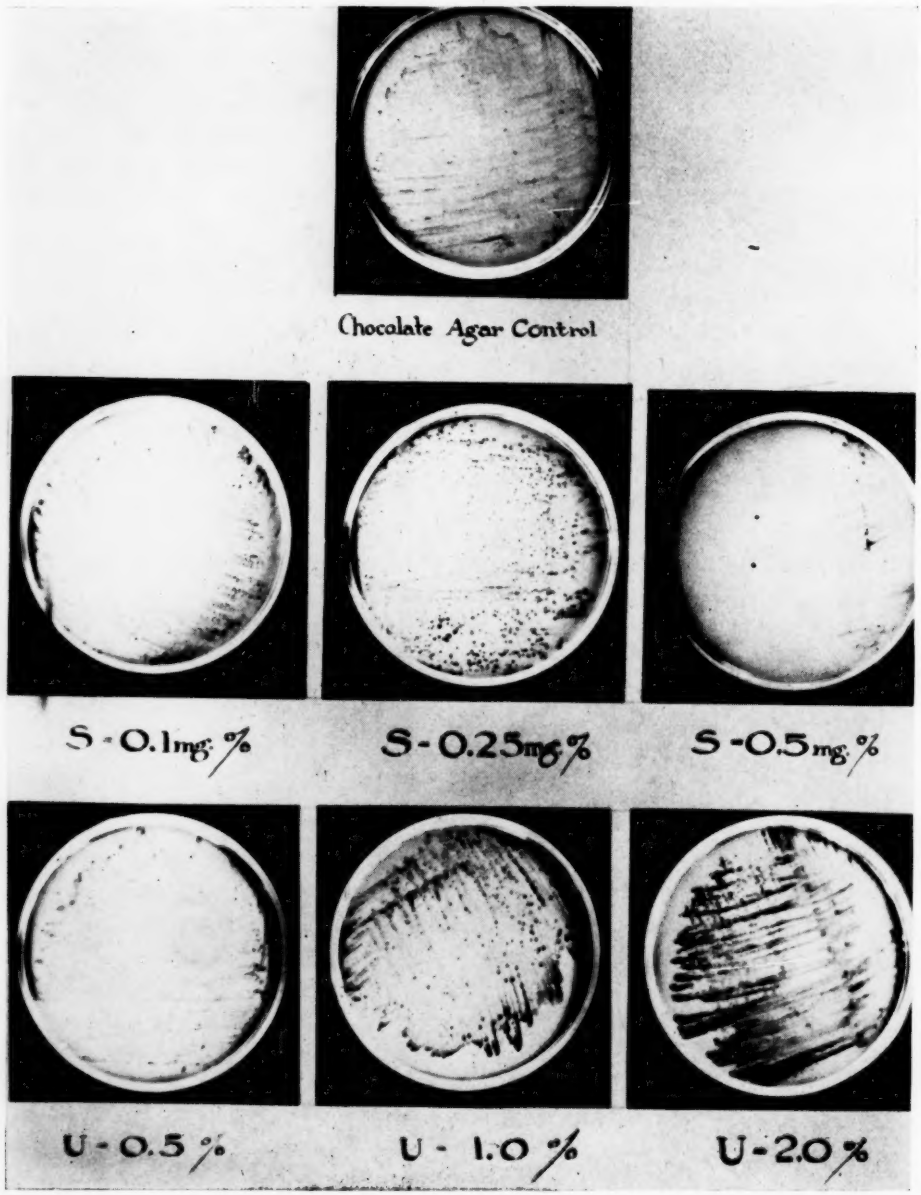

Fig. 1.—Sulfonamide-resistant gonococci on chocolate agar, chocolate agar with sulfathiazole and with urea. S = Sulfathiazole. U = Urea.

urea alone in the doses used has no bacteriostatic or bacteriolytic effect on gonococci in vitro or in vivo.

Of interest in Fig. 2 is a definite tendency to diminished growth of gonococci in increasing strengths of combined urea and sulfathiazole when compared to the same strengths of either substance alone. The three strengths of sulfathiazole with 2.0 per cent urea showed no growth. This gives the appearance of a potentiating effect.

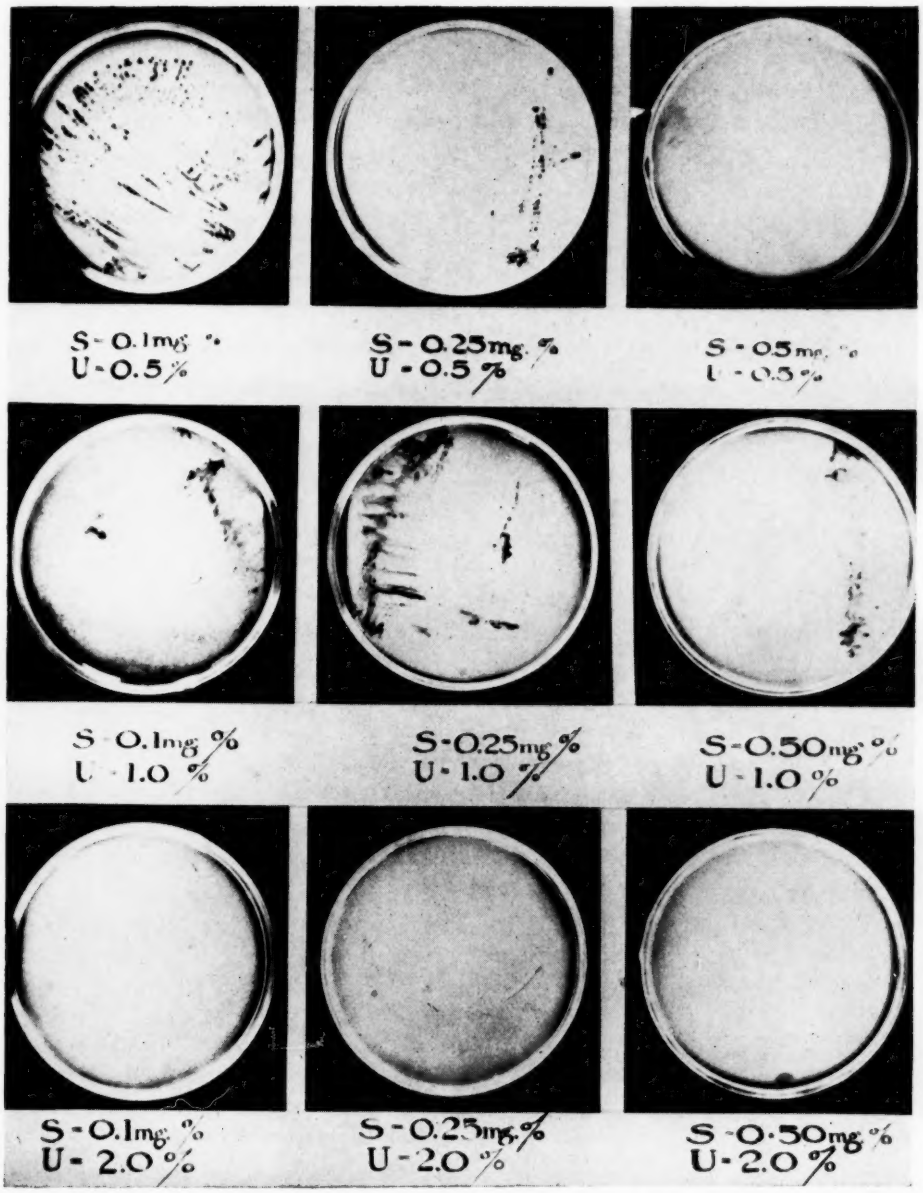

Fig. 2.—Sulfonamide-resistant gonococci on chocolate agar with various concentrations of sulfathiazole and urea. S = Sulfathiazole. U = Urea.

DISCUSSION

The modus operandi of urea and a sulfonamide against organisms showing some degree of resistance to previous sulfonamide applications is not clearly understood at the present time. It seems of interest that whereas the external application of urea and a sulfonamide has been found to be beneficial against certain sulfonamide-resistant organisms,[4, 8, 9] in our study the internal administration of urea and a sulfonamide has seemed to have a certain effectiveness against gonococci previously resistant to oral sulfonamides alone. Whether this is a synergistic effect (? potentiating) or whether it is an action mediated through other chemical changes is not known.

It is generally understood that different strains of gonococci may show varying degrees of resistance to the sulfonamide drugs. It may be that our patients who failed to respond to the urea-sulfonamide regime had more resistant organisms than the cases that did respond satisfactorily. Further, it would have been desirable to treat the nine cases that failed to respond to large doses of sulfathiazole on regular doses of sulfathiazole and urea. Since our study had to be terminated by the directions for us to use penicillin in all sulfonamide-resistant cases, studies in that direction could not be carried out. To nine of the failures in our series we gave penicillin with prompt recovery in each case.

The use of urea with a sulfonamide, however, may serve a useful purpose for the treatment of sulfonamide-resistant gonorrhea where penicillin is not available. Urea-sulfonamide treatment has several advantages—easily administered (the patient can be treated in the field on an ambulatory status and need not be hospitalized), no significant toxic effects, and very inexpensive.

SUMMARY AND CONCLUSIONS

At a time when penicillin was not yet available for the treatment of sulfonamide-resistant gonorrhea, we became interested in trying urea by mouth with a sulfonamide in a series of soldiers with resistant gonorrhea at the Valley Forge General Hospital. Our interest in this direction was aroused by several reports of success, both in vitro and in vivo, in which urea and a sulfonamide were used against certain sulfonamide-resistant organisms. Whereas the clinical benefits that had been reported from the use of this combination had resulted from its external application, we were desirous to see if it produced any benefits when applied internally.

To 40 males whose gonorrhea had failed to respond to sulfonamide treatment as prescribed routinely in the Army, we gave the following course of treatment: 30.0 Gm. urea by mouth daily for three days, 30.0 Gm. urea and the same sulfonamide in the same dosage on which the patient had failed previously for three days, then the sulfonamide alone for three days. Sulfathiazole was used in 38 cases, sulfadiazine in 2. Certain control studies were carried out.

As a result of this study we present the following conclusions:

1. Urea and a sulfonamide orally appear to be effective in certain cases of gonorrhea that have failed to respond to a sulfonamide alone. Of 40 cases so treated, 21, or 52.5 per cent, were cured; 19, or 47.5 per cent, failed to respond satisfactorily.

2. Comparative studies of the successful results and the failures, in terms of the number of previous attacks of gonorrhea, lag in the institution of initial treatment, and number of previous courses of sulfonamide therapy, gave no clue to the reasons for failure.

3. Urea alone, 30.0 Gm. a day, continued for as long as seven days, had no effect on the gonorrhea.

4. Sodium bicarbonate with a sulfonamide, given to prevent renal toxic effects, was no more effective in combatting the gonorrhea than the same dosage of sulfonamide alone.

5. To 15 patients double the usual dose of sulfonamide was given alone to satisfy ourselves that what was being called "sulfonamide-resistant" was not due alone to too small dosage. Only 3, or 20 per cent, of the patients thus treated responded satisfactorily. Four patients who failed on this treatment were placed on urea and sulfonamide in regular doses and two responded satisfactorily. Two others who had failed on large doses of sulfonamide alone were given another course of large doses of sulfonamide and 30.0 Gm. urea daily and one responded successfully.

6. Twelve of the failures on urea and sulfonamide were given anterior urethral instillations of silver salts. Eight responded successfully, 4 did not. The impression was gained that those patients who responded did so much more rapidly on this treatment after a urea-sulfonamide regime than similar patients who had received sulfonamide alone.

7. To 9 of the series of 40 patients penicillin was given with an excellent response in each instance.

8. The administration of urea and a sulfonamide by mouth may be effective in the treatment of patients with gonorrhea previously resistant to sulfonamides alone. The treatment is not as effective as penicillin therapy; however, it may be of usefulness where penicillin is not available. The administration of urea-sulfonamides is easy—the patient can be treated on an ambulatory status and need not be hospitalized. There are no more toxic effects than with sulfonamides alone, and the treatment is very inexpensive.

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ACUTE AGRANULOCYTOSIS DURING SULFAMERAZINE THERAPY

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THE general use of the various members of the sulfonamide drugs has been followed by reports of toxic manifestations involving the blood and blood-forming organs. Acute agranulocytosis has been observed in patients to whom sulfanilamide,[1, 4] sulfapyridine,[1, 2, 4] sulfathiazole,[3, 4] sulfadiazine,[5] and succinylsulfathiazole were administered.[6] The following case report adds sulfamerazine to this list.

REPORT OF CASE

S. M., a white male, 51 years of age, was hospitalized because of a neck injury. On admission, in addition to his injury, he had an acute pharyngitis with a fever of 104.1° F., pulse 120, and respiration 22. His past history was irrelevant. Examination revealed a poorly nourished individual. All motions of the neck were restricted by muscle spasm. The pharynx was injected and ruddy in appearance. Diffuse coarse râles were heard over the tracheal area. The blood pressure was 128 systolic and 78 diastolic. The remainder of the physical examination was negative. Examination of the blood showed the hemoglobin to be 16 Gm. per 100 c.c. of blood, red blood cell count 4,750,000 per c.mm., and the white blood cell count 8,800 per c.mm. Differential showed 76 per cent polymorphonuclears, 22 per cent lymphocytes, and 2 per cent monocytes. The urinalysis was essentially negative.

The patient was given 0.5 Gm. of sulfamerazine twice during the first day and three times daily thereafter for fourteen days. The temperature returned to normal within twenty-four hours, and his general condition appeared normal. Because of the neck injury, a "Queen Anne" plaster cast collar was applied. The following twelve days were uneventful. On the fifteenth day after admission, the patient developed a tracheobronchitis and the dose of sulfamerazine was increased to 1 Gm. three times daily. No other drugs were administered. Symptoms subsided materially after two days, but the temperature fluctuated between normal and 101° F. for the following six days. A blood count at this time revealed hemoglobin, 14 Gm.; red blood cells, 4,350,000; white blood cells, 3,000; polymorphonuclears, 7; and lymphocytes, 93. Because of neutropenia, the drug was discontinued. Forty-two grams were administered during the 23 days.

On the following day the patient's general condition became worse. Respirations were faster. The appearance of a cough, with blood-tinged sputum on one occasion, indicated that there had been an exacerbation of the tracheobronchitis. A roentgenogram of the lungs on this day showed an intensification

From the West Jersey Hospital, Camden, N. J.
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TABLE I

DATE	HEMOGLOBIN GM./100 c.c.	ERYTHROCYTES PER C.MM.	LEUKOCYTES PER C.MM.	MYELOBLASTS %	MYELOCYTES %	METAMYELOCYTES %	STAB CELLS %	NEUTROPHILES %	EOSINOPHILES %	BASOPHILES %	LYMPHOCYTES %	MONOCYTES %	MAXIMUM DAILY TEMP. F.	LIVER EXTRACT C.C.	PENTNUCLEOTIDE C.C. (INTRAMUSCULARLY)
12/5	16.0	4,750,000	8,800	--	--	--	--	76	0	0	22	2	----	--	--
12/27	14.0	4,350,000	3,000	--	--	--	--	7	0	0	93	0	100.3	--	--
12/28	14.4	4,110,000	1,200	0	0	0	0	0.5	0	0.5	99	0	104.1	--	40
12/29	14.3	3,970,000	1,400	0	0	0	0	0	0	0	100	0	104.2	7	40
	---	--------	2,200	2	3	0	0	0	0	0	94	1		--	--
12/30	14.0	4,330,000	2,200	0	3	0	0	0	0	1	96	0	104.4	6	30
	---	--------	1,800	--	--	--	--	--	--	--	--	--		--	
12/31	13.5	3,990,000	600	1	6	0	0	0	0	0	90	3	104.0	2	20
1/2	13.9	3,900,000	----	--	--	--	--	--	--	--	--	--	99.3	--	40
1/3	12.8	3,850,000	11,600	3	5	0	0	0	0	0	90	2	98.0	--	20
1/4	12.0	3,800,000	18,800	2	4	17.5	16.5	45	2	0	13	0	100.2	--	10
1/5	13.3	4,250,000	17,500	--	--	--	--	77	0	0	21	2	100.0	--	--
1/6	---	--------	25,000	--	--	--	--	90	0	0	10	0	102.4	--	--
1/8	11.2	3,570,000	25,600	--	--	--	--	90	0	0	9	1	100.0	--	--
1/11	13.5	4,200,000	13,100	--	--	--	--	82	0	0	18	0	98.0	--	--
1/13	11.2	3,650,000	10,300	--	--	--	--	80	0	0	20	0	98.3	--	--

of the hilar and adjacent pulmonary markings and was interpreted as a tracheobronchitis by the roentgenologist, Dr. E. C. Shull. The white blood cell count fell to 1200 per c.mm., with 1 per cent polymorphonuclear leucocytes, and 99 per cent lymphocytes. Therapy with pentnucleotide and liver extract (Armour's concentrated extract, 10 U.S.P. units per c.c.) was instituted as shown in the accompanying table. Forty-eight hours after the termination of sulfamerazine administration, the blood level of the drug was 4.1 mg. per 100 c.c. of blood.

During the following four days, the patient's condition remained serious. The white blood cell count fluctuated between 600 and 2,200 cells per c.mm.; there was a total absence of all granulocytes on one occasion. The hemoglobin and red blood cell levels became moderately reduced. Although the platelets were not counted, they appeared plentiful in the blood smears. There were 0.4 per cent reticulocytes. The fever varied from 102° to 104° F. during this period. The pulmonary signs diminished. There was a mixed bacterial flora in the sputum but pneumococci predominated. The latter did not type by the Neufeld method. Diarrhea and vomiting appeared. The icterus index was 5. Abdominal examination was negative. There were periods of mental disturbances during which the patient became irrational. The urine showed a trace of albumin. During the next three days, the symptoms subsided materially and the blood picture improved rapidly. The number of white cells increased and for several days there was a leucocytosis of 25,000 with a polymorphonuclear count of 90 per cent. Within two weeks the blood had returned to normal and has remained so for two months following the patient's discharge from the hospital.

COMMENT

The patient received 42 Gm. of sulfamerazine in 22 days. The agranulocytosis appeared during the same period of drug administration commonly reported for the other sulfonamides. The clinical picture of upper respiratory infection followed by vomiting and diarrhea was preceded by leucopenia and neutropenia. The subsequent agranulocytosis was accompanied by an aggravation of all symptoms. It is unlikely that the infection *per se* was responsible for the agranulocytosis. The symptoms seemed out of all proportion to the physical signs. In all likelihood, the drug-induced agranulocytosis aggravated or helped to precipitate the infection. Pentnucleotide and liver extract were given in large doses. It is impossible to tell whether their administration influenced the course of the disease favorably. Improvement followed five days after their initial administration and six days after sulfamerazine was discontinued. Forty-eight hours after administration of the drug was stopped, the blood still showed 4.1 mg. of sulfamerazine per 100 c.c. of blood.

SUMMARY

A case of agranulocytosis occurring in an adult male who was receiving sulfamerazine therapy is reported. The dyscrasia appeared after twenty-two days of daily administration of the drug. A total dose of 42 Gm. was given. Recovery followed the termination of sulfonamide therapy and treatment with large doses of pentnucleotide and liver extract.

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ISOLATION OF BACTERIUM TULARENSE FROM THE SPUTUM OF AN ATYPICAL CASE OF HUMAN TULAREMIA

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THE early diagnosis of typhoidal or pneumonic tularemia depends on the isolation of *Bacterium tularense*.[1] A positive blood culture can be obtained, in some cases, by the use of special media such as blood dextrose cystine agar, but bacteriemia has not been demonstrated with regularity except in the terminal stage of fatal cases. There have been few attempts to isolate the organism from the secretions of the respiratory tract, chiefly, no doubt, because of the paucity of sputum and lack of clinical signs of respiratory infection. In the course of an experimental study of tularemia in dogs it was noted that *B. tularense* exhibited a particular affinity for the mucosa of the upper respiratory tract and in some instances persisted in the nasopharynx after clinical recovery.[2] Isolation of the organism was accomplished by mouse inoculation and subsequent culture on blood dextrose cystine agar.

A study was therefore made to see whether *B. tularense* exhibited a similar predilection for the respiratory tract in human infections.

CASE HISTORY

S. L., a twelve-year-old white schoolgirl, was admitted to the Maxwell Field Post Hospital on May 6, 1943, because of persistent fever of unknown etiology. The presenting symptoms were fever and sore throat. She gave a history of having had fever since April 24, 1943. A local physician had prescribed quinine, but the girl had derived no benefit from the drug. Her oral temperature had ranged from 100° to 103.4° F. She did not look sick. Results of physical examination were essentially negative, except that a slight enlargement of the spleen was detected. There was evidence of subacute inflammation of the pharynx, but this did not appear significant. A chest x-ray examination gave negative results. Agglutination tests were run for undulent fever, typhoid, typhus, and tularemia. The blood serum agglutinated *B. tularense* antigen through a dilution of 1:320. The other agglutination tests were negative.

A review of the girl's history revealed that some children in the neighborhood had found a litter of young wild rabbits on April 18, 1943, and that the girl had played with these rabbits. She had tried to keep the rabbits alive by artificial feeding, but all of them died.

The girl was discharged from the hospital on May 16, 1943. She was kept from having contact with other children but otherwise was allowed ordinary activity. Her temperature was normal except for occasional slight fever in the late afternoon.

METHOD OF STUDY

Owing to the presence of an inflammatory process in the pharynx and the lack of other localizing signs it appeared probable that the throat was the site of the primary lesion. In order to find out whether the patient was still harboring *B. tularense* and might be a potential source of infection to others, an attempt was made to isolate the organism. The first specimen studied consisted of material obtained from a throat swab taken on May 11, the seventeenth

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day of illness. This was immersed in 4 c.c. of veal infusion broth and sent to the laboratory of the Alabama State Board of Health, where 0.5 c.c. of the broth suspension was injected intraperitoneally into each of two white mice. The mice were prostrate on the fourth day, and, when killed, showed typical lesions of tularemia in the liver and spleen. A pure culture of *B. tularense* was obtained on blood dextrose cystine agar (Difco) from the mouse spleen, and the organism was agglutinated out to full titer with a known positive serum.

As the first specimen proved positive, another specimen was requested. This was obtained on May 19, the twenty-fifth day of illness. It consisted of a small amount of sputum which looked like normal saliva. This sputum was diluted with an equal amount of distilled water and four mice were injected intraperitoneally, each receiving 0.5 c.c. of the suspension. All of the inoculated mice developed tularemia.

A third suspension of sputum was obtained on May 24, the thirtieth day of illness. Four mice were inoculated intraperitoneally with this material as noted above. Again all four mice developed tularemia. A pure culture was obtained on blood cystine agar from mouse spleen, and the identity of the organism was proved by its agglutination to full titer with a known immune serum.

Sputum obtained on May 29th, the thirty-fifth day of illness, was negative by mouse inoculation, as was also the material obtained from a throat swab taken on June 6. On June 24 we received some scrapings from a yellow membraneous lesion on the girl's right anterior pharyngeal pillar. The scrapings were placed in broth immediately after they were obtained. The material was triturated and injected intraperitoneally into mice and guinea pigs, but these animals remained well.

EPIDEMIOLOGICAL STUDIES

It was found that nine of the girl's playmates had also handled the young rabbits. Blood specimens were taken from these children on June 24. Agglutination tests performed on the sera were all negative. The blood sera of three dogs from a near-by farm were also tested. One of these dogs gave a positive agglutination with *B. tularense* antigen in a serum dilution of 1:80.

OBSERVATIONS AND DISCUSSION

It is obvious that the diagnosis of tularemia would not have been made in this case if agglutination tests had not been performed. The illness would have been classed as fever of undetermined origin. The history of contact with rabbits was not elicited until the correct diagnosis had been made. The patient had no primary lesion on either hand, nor was there axillary adenopathy. This suggests that infection took place by way of the mouth or nasopharynx. Cases similar to this one have been reported by Anschuetz[3] and Waddell and Birdsong.[4]

It is possible that tularemia could be spread from person to person by inhalation of infected droplets resulting from coughing or sneezing. Although tularemia shows little ability to pass from person to person, should it once achieve this, an alteration of tropism might lead to greater infectiousness by this route. Kennedy[5] reported a fatal case of pulmonary tularemia in a young

girl with no history of contact with rabbits or exposure to ticks. There are numerous records of cases of tularemic pneumonia without any evident portal of entry. Blackford[6] recommends testing the sera of all persons with unusual pulmonary lesions for *B. tularense* agglutinins, since several unsuspected cases of tularemia have been discovered by him in this way.

The diagnosis of tularemia must be considered in cases of cryptogenic fever and atypical pneumonia. Agglutinins do not develop in the blood serum until the second or third week of the disease; the early diagnosis of tularemia, therefore, depends on the isloation of the infecting organism from the blood, from the primary lesion if present, or from the sputum in cases of tularemic pharyngitis and pneumonia. The only satisfactory method at present for proving the existence of the organism in the sputum or nasopharynx is animal inoculation. The mouse appears to be a particularly suitable animal for this purpose. As the sputum is apt to be scanty in cases of tularemic pharyngitis and pneumonia, it will usually be best to collect specimens with a swab. Swabs of both the nasopharynx and throat should be taken, and these should be immersed in a small amount of distilled water or veal infusion broth. The suspension of the material from the swab may then be inoculated intraperitoneally into mice or guinea pigs.

SUMMARY

This report describes the isolation of *B. tularense* from the sputum of a child with a nonfatal case of tularemia. The patient was a twelve-year-old girl, who was admitted to the hospital because of unexplained fever. *B. tularense* was isolated by mouse inoculation on three occasions from the seventeenth to the thirtieth day after the onset of the disease. The identity of the organism was proved by culture and cross agglutination tests with known strains of *B. tularense*. The last two isolations were made after the patient was discharged from the hospital and had resumed normal activity.

The persistence of *B. tularense* in the respiratory tract indicates that there is a potential danger of contact infection. The white mouse is recommended as a suitable laboratory animal for diagnostic inoculation studies in suspected cases of tularemia.

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NATURAL OCCURRENCE OF TULAREMIA IN DOGS USED AS A SOURCE OF CANINE DISTEMPER VIRUS

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THE use of a live virus vaccine for the immunization of animals requires especial care in order to avoid spreading other diseases accidentally derived from the animals used as a source of virus. Dogs may be naturally infected with the virus of lymphocytic choriomeningitis,* and this disease may be maintained as an inapparent infection in the course of serial passage of canine distemper virus.[1] The following report describes the isolation of *Bacterium tularense* from dog spleen tissue used for the production of canine distemper vaccine.

There are accounts, on record, of dogs having developed tularemia in nature.[2, 3] The diagnosis has depended on the development of an acute illness following contact with wild rabbits and the subsequent demonstration of a high serum agglutination titer for *B. tularense*. The natural susceptibility of the dog to tularemia has been questioned, because the causative organism has not been isolated from the blood or tissue of a naturally infected dog and properly identified. Tularemia infections have been reported in man following the bite or scratch of cats.[4] The study of tularemia and its course in experimentally infected dogs, cats, and other animals has revealed some information which may be of value; the pertinent findings are presented herewith.

MATERIALS AND METHODS

In the course of a study of canine rabies it has been necessary to protect the experimental animals from intercurrent infections, notably canine distemper. The usual procedure has been to give one subcutaneous injection of a formalin inactivated dog spleen tissue vaccine, followed four to six weeks later by an injection of active distemper virus.

The specimen from which *B. tularense* was obtained consisted of a pool of spleen tissue from twenty-five puppies that were killed at the time of maximum febrile response following a simultaneous natural and experimental exposure to canine distemper at a commercial biological laboratory. The puppies were from farms and villages in an area where tularemia is endemic among wild rabbits. Each puppy was given a subcutaneous injection of 1 c.c. of a 20 per cent suspension of spleen tissue from a previous passage of the canine distemper virus. The puppies were then kept in a distemper kennel where there was a high likelihood of superimposed natural exposure. A normal ferret was given a subcutaneous injection of 1 c.c. of a 20 per cent suspension of the dog spleen pool noted above and was sent to this laboratory by Railway Express. The animal was received the following day. An initial febrile reaction developed on the second day after inoculation, and for a period of forty-eight hours the rectal temperature varied from 102.3° to 104.7° F. By the seventh day the tempera-

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*Lymphocytic choriomeningitis virus has been isolated from commercial canine distemper virus specimens at this laboratory.

ture was normal. There was a second febrile period beginning on the eighth day, reaching a maximum on the eleventh day, when a rectal temperature of 107.9° F. was recorded. The ferret was by this time obviously sick, refusing food and exhibiting a mucopurulent lacrimal and nasal discharge. The ferret was killed on the thirteenth day after inoculation. Tissue was taken for histological study, and the entire spleen was saved as a source of virus.

The course of infection was typical of that produced by canine distemper in ferrets except for the occurrence of a small abscess at the site of inoculation. At the time of autopsy a smear was made from the contents of the abscess and stained by Gram's method. No bacteria could be identified. The gross postmortem examination was negative except for moderate enlargement of the liver and spleen. The lungs appeared normal. Paraffin sections were prepared from tissue fixed in acetic Zenker's solution and stained with eosin-methylene blue according to the method of Mallory, but modified in this laboratory to secure better staining of the intracytoplasmic inclusion bodies of rabies and canine distemper. Ethyl eosin (alcohol soluble) adjusted to a pH of 4.5 with acetic acid was used for the primary stain, followed by Unna's alkaline methylene blue stain. There were intracytoplasmic inclusion bodies in the epithelial cells of the bile ducts and in the medullary cells of the adrenal glands consistent with those ordinarily found in canine distemper. Sections of the liver, however, presented an unusual picture due to the presence of miliary foci of coagulative necrosis. We were unable to demonstrate bacteria in paraffin sections of the liver stained by the carbolfuchsin-methylene blue technique for acid-fast organisms or by Gram's method for bacteria.

A portion of the spleen from the ferret was triturated in a mortar and diluted to a 10 per cent suspension in distilled water. The suspension was centrifuged, and four mice inoculated intracerebrally with the supernate. We have followed this procedure routinely at the laboratory in order to avoid introducing lymphocytic choriomeningitis into our dog kennels. On the third day after injection all four of the inoculated mice were sick. They exhibited a mucopurulent lacrimal discharge, their backs were humped, and their furs were ruffled. Two were killed seventy-two hours after inoculation, and the other two died the next day. Smears and impressions of the brain tissue showed an acute meningoencephalitis, with a scanty cellular exudate in the meninges. No bacteria could be identified in preparations stained by Gram's method. Cultures made on blood agar and in veal infusion broth, enriched by the addition of whole rabbit blood to 10 per cent, were negative. Preliminary studies showed that the agent was infectious for mice by intranasal, subcutaneous, and intraperitoneal inoculation. Dogs, guinea pigs, rabbits, hamsters, and baby chicks were all highly susceptible, and a uniformly fatal infection resulted within three to five days after intracerebral inoculation. A variety of bacteriological media were used in an attempt to cultivate the infectious agent, including blood dextrose cystine agar. No growth was obtained, and therefore the agent was thought to belong to the virus group of diseases. A rather extended study was made before the infectious agent was proved to be *B. tularense*.

The inoculum for testing the susceptibility of the various species studied was prepared as follows: Infected tissue was weighed, ground in a mortar with crystalline alundum, and diluted to a 10 per cent suspension by weight with

distilled water. This suspension was centrifuged for ten minutes at 2,500 r.p.m., and the supernate was saved as a source of the infectious agent. In the titration studies, serial decimal dilutions of the stock 10 per cent supernate were made by serially transferring 1 c.c. to 9 c.c. of distilled water. For the neutralization tests a pretitrated stock suspension kept at −70° C. was thawed, and serial decimal dilutions were prepared in distilled water containing 10 per cent human serum. The dilutions were so planned that they contained 1, 10, 100, and 1,000 50 per cent MLD's per 0.03 c.c. when diluted with an equal volume of test serum. This mixture was incubated at 37° C. for one hour and then injected intracerebrally into mice.

The agglutination tests were performed according to the method recommended by the National Institute of Health. The end point was taken as the highest dilution of serum giving a 4+ agglutination reaction.

IDENTIFICATION STUDIES

We soon learned that the agent was not related to canine distemper. Dogs and ferrets immune to canine distemper were fully susceptible to infection. Sera known to be fully active in neutralizing the viruses of lymphocytic choriomeningitis, mouse pneumonia, meningopneumonitis, human influenza, canine distemper, and equine encephalomyelitis were tested by Dr. Frank L. Horsfall, Jr. for their power to neutralize the agent in vitro. In no instance was there any evidence of neutralization. We were also unable to demonstrate any evidence of neutralizing substance in the sera of vaccinated dogs and rabbits previously tested and found immune.

It was noted that tiny coccoid bodies could be demonstrated in large numbers in the spleens of infected mice in impressions stained with Giemsa's stain. These bodies were basically intracellular but were also present in large numbers outside of cells, evidently as the result of disintegration of parasitized cells. They could not be demonstrated with certainty in impressions stained by Gram's method. Impressions of the brain of intracerebrally inoculated mice stained by Giemsa's method showed occasional parasitized cells. The bodies were more numerous in impressions of liver tissue but not as abundant as in the spleen. The bodies appeared blue in preparations stained with the Macchiavello stain.

The first proof of the identity of the agent isolated from the dog spleen material was obtained when the blood of dogs convalescent from infection produced by intramuscular inoculation was tested for agglutinins to *B. tularense*. The blood of three dogs tested three months after inoculation had titers of 1:40, 1:80, and 1:2560 respectively with *B. tularense* antigen, and no agglutination was obtained with control bloods from normal dogs.

Cultures were again made on blood dextrose cystine agar. Inoculations were made with loopfuls of spleen material from infected mice. These were again negative. No growth was obtained in veal infusion broth enriched with 10 per cent whole rabbit blood, in Brewer's liquid media containing sodium thioglycolate and methylene blue,[5] or on blood agar plates. The Brewer's media with whole rabbit blood added to a concentration of 10 per cent was also used with negative results. Cultures were tested for infectivity by mouse inoculation after seven to ten days' incubation, but this material failed to reproduce the disease. A batch of blood dextrose cystine agar was then prepared from de-

hydrated media (Difco) as recommended by Ransmeier.[6] We readily obtained growth of a gram of negative coccobacillus on this media. The colonies had the typical characteristics of *B. tularense*, and the identity of the organism was determined by animal inoculation and agglutination by a known positive serum.

Though the blood dextrose cystine agar originally used was unsatisfactory for isolating the organism from infected tissue, it was adequate for maintaining an established avirulent laboratory strain of *B. tularense*.

A culture of the organism isolated at this laboratory was sent to Dr. Edward Francis of the National Institute of Health who reported that the organism had the typical morphology and animal pathogenicity of *B. tularense*. It was agglutinated out to full titer with a known antitularense serum.

TULAREMIA IN DOGS

Dogs have been inoculated intradermally, subcutaneously, intramuscularly, intranasally, and intracerebrally with suspensions of infected tissue. A few animals were exposed by feeding tissue infected with *B. tularense*. In all instances the animals exhibited evidence of a systemic infection. The incubation period and course of the disease varied with the type of exposure.

Intradermal Inoculation.—Two 4-month-old dogs were given 0.1 c.c. of a 10 per cent suspension of infected mouse brain into the skin of the upper lip on each side of the nose. Both developed fever forty-eight hours after inoculation. The cervical lymph nodes became enlarged on the third and fourth days, coinciding with the development of pustules at the site of injection. One of these dogs was killed twenty-eight days after exposure, and mice were injected intraperitoneally with a suspension of tissue from the inoculation site and regional lymph nodes. The mice all developed tularemia. Blood taken from this dog on the twenty-eighth day agglutinated *B. tularense* antigen in a serum dilution of 1:80. The other dog was bled three months after exposure and had a blood serum agglutination titer of 1:20 with *B. tularense* antigen.

Subcutaneous Inoculation.—Seven mature dogs were injected subcutaneously with 0.25 c.c. of a 10 per cent suspension of infected mouse brain. All developed fever within forty-eight hours. The rectal temperature rose from a normal level of 100-102° F. to 103-105° The animals also developed a watery lacrimal and nasal discharge. The temperature returned to normal from the third to fifth day. A local area of induration developed at the site of injection on the second to fourth day, and progressed to abscess formation. These abscesses opened spontaneously discharging a serosanguineous fluid. From the seventh to fifteenth day, vesiculopapular skin lesions appeared in the axillary and inguinal regions and over the abdomen. The dogs developed a secondary febrile period on the seventh to eighth days which lasted two to three days. At this time they showed a mucopurulent lacrimal and nasal discharge. They were by this time obviously ill. Two of the dogs had fever on occasions as late as twenty days after inoculation, but all recovered.

Puppies were found to be more susceptible than older dogs. Four 3-month-old puppies were given a subcutaneous injection of 0.25 c.c. of a 10 per cent suspension of infected mouse brain. All four of them developed high fever. One of them died on the eleventh day and another on the twelfth day. Autopsies showed focal necrosis of the liver and spleen and extensive pneumonia.

The infecting organism was recovered from the pneumonic lung in both instances by mouse inoculation. A total of twelve puppies, approximately three months of age, were infected by subcutaneous inoculation. They all developed a severe illness, and three died of the infection. The puppies had higher and more protracted fever than older dogs.

Intramuscular Inoculation.—Four dogs, nine to thirty-three months of age, were injected intramuscularly with 0.5 c.c. of a 10 per cent suspension of infected mouse spleen. They ran a course similar to those inoculated subcutaneously, but the illness was more severe and protracted. One of the dogs had a serum agglutination titer of 1:2560 with *B. tularense* antigen when bled two months after injection. At five months the titer was 1:1280, and at ten months it was 1:320. When bled at two months, the other dogs had titers of 1:40 and 1:80.

Intranasal Inoculation.—Six dogs, twelve to twenty-four months old, were inoculated intranasally with 0.25 c.c. to 0.5 c.c. of a 10 per cent suspension of infected mouse brain. These dogs developed fever three to four days after exposure. One 12-month-old dog had a rectal temperature of 107.5° on the twelfth day, and another of the same age had a temperature of 106° F. on the tenth day. Both of these dogs were killed on the sixteenth day. Autopsies showed focal necrosis of the liver and enlargement of the spleen. The upper respiratory passages were congested and covered with a mucopurulent exudate. There were a few small areas of early pneumonia. The other dogs recovered. One of the dogs was bled one month after exposure. The blood serum had a titer of 1:160 with *B. tularense* antigen. Two of the dogs were bled six months after exposure. One had a positive agglutination reaction in a dilution of 1:20, while the other was entirely negative.

Two additional dogs were inoculated intranasally with 0.25 c.c. of a 10 per cent suspension of infected mouse brain. Both developed fever, enlarged cervical lymph nodes, and watery lacrimal and nasal discharge three to four days after exposure. On the eighth to tenth day the discharge from the eyes and nose became mucopurulent. Beginning on the fifth day, specimens of nasal secretion were taken by introducing cotton swabs into the nasopharynx. The cotton swabs were then immersed and rotated in 2 c.c. of distilled water. Four mice were given an intraperitoneal injection of 0.25 c.c. of this suspension. Similar specimens were taken at intervals until two negative results were obtained. The nasal secretion from both dogs was positive for *B. tularense* by mouse inoculation on the fifth day. Positive specimens were obtained from one dog through the twenty-fifth day and from the other through the twelfth day. These dogs were twelve and twenty-four months old respectively. The older dog carried the organism for the longer period, and appeared normal for ten days preceding the last instance of recovery of the organism. This dog, one month after inoculation, had a serum agglutination titer of 1:160 with *B. tularense* antigen.

Intracerebral Inoculation.—Four dogs were injected intracerebrally with 0.25 c.c. of a 10 per cent suspension of infected mouse brain. All developed high fever twenty-four hours after injection. Two died on the second day. The other two were dead by the fourth day.

Feeding Infected Tissue.—One 8-month-old dog was fed three mice infected with tularemia. The dog became sick forty-eight hours later. The temperature rose to 104.4° and the eyes and nose showed a mucopurulent discharge. The animal was obviously ill. After an acute illness of five days it gradually improved. Blood was taken daily for mouse inoculation and for culture on blood dextrose cystine agar. We failed to isolate *B. tularense* from the blood. A few specimens of the nasal secretion were taken by the nasopharyngeal swab method. *B. tularense* was isolated from the nasopharynx as late as the eighteenth day after the dog had eaten the infected mice. The isolation was checked by agglutination tests. Blood taken on the tenth day after exposure gave a 4+ agglutination reaction with *B. tularense* antigen in a dilution of 1:80. The titer rose to 1:640 on the fifteenth day. By the end of three months the titer had dropped to 1:80. Two normal dogs kept in the same pen with this dog failed to develop fever or other evidence of infection. Neither of these dogs developed agglutinins to *B. tularense*.

Pathology.—The dogs that died or were killed during the secondary febrile period all showed small areas of focal necrosis in the liver and enlargement of the spleen. In several instances the spleen also showed pin-point size areas of focal necrosis. There was enlargement of the lymph nodes related to the site of exposure, and these also contained areas of focal necrosis. The lungs showed slight to extensive pneumonia. Paraffin sections of pneumonic lung stained by the Wolbach modification of Giemsa's stain revealed enormous numbers of bacteria consistent in appearance with *B. tularense*. Some alveoli were completely filled with tiny coccoid organisms. Small clumps of bacteria were also found in the sinusoids of the liver. This explains the occurrence of areas of focal necrosis in all parts of the liver lobule. Bacteria could not be demonstrated in the areas of focal necrosis. These foci presented a coagulative type of necrosis with general loss of cell detail.

Despite the rapid and fatal course following intracerebral inoculation, there was no gross evidence of meningitis other than marked vasocongestion. It was usually difficult to find the site of injection. Microscopic examination revealed a scanty inflammatory exudate in the meninges composed primarily of polymorphonuclear cells. There was no deposition of fibrin.

Tissue Infectivity.—Various tissues taken from dogs that died or were killed during the second febrile period were tested for infectivity by mouse inoculation. The infecting organism was recovered from the inoculation site, regional lymph nodes, and spleen. The liver was not tested because it was obvious from the gross appearance that it would be positive. The submaxillary gland did not prove infectious by mouse inoculation.

TULAREMIA IN CATS

Subcutaneous Inoculation.—Two young cats were inoculated subcutaneously with 1 c.c. of a 10 per cent suspension of the ferret spleen material. They developed fever of 104.6° to 106.6° F. forty-eight hours after inoculation. Fever subsided in from forty-eight to seventy-two hours, but the animals appeared sick for two weeks after injection. When reinoculated one month later they exhibited no fever or symptoms of infection. Two young cats were given a subcutaneous injection of 0.25 c.c. of a 10 per cent suspension of infected mouse brain and

were killed on the fourteenth day after inoculation. There were several lobular areas of pneumonia, and the infecting agent was recovered from the lung tissue by mouse inoculation.

Intranasal Inoculation.—Two young cats given 0.1 c.c. of a 10 per cent suspension of infected mouse brain intranasally were killed on the fourteenth day after infection. These also had lobular areas of pneumonia, and the infecting agent was recovered from the lung tissue by mouse inoculation. The cats killed on the fourteenth day after inoculation all showed focal necrosis of the liver and enlargement of the spleen.

Intracerebral Inoculation.—Two adult cats were given 0.1 c.c. of a 10 per cent suspension of infected mouse brain by intracerebral inoculation. Both developed high fever and incoordination twenty-four to forty-eight hours after injection. One died on the eighth day but the other recovered. The cat that recovered was very sick for a period of five days. It was hardly able to get up, a mucopurulent secretion exuded from the eyes and nose, and it appeared apathetic and sleepy.

TULAREMIA IN MICE

Brain tissue from mice infected by intracerebral inoculation was used as a source of the infecting agent for most of the susceptibility experiments, although the spleen contained a somewhat higher concentration of organisms per gram of tissue. Titrations of infected brain gave a titer of at least 0.03 c.c. times 10^{-8} when inoculated either intracerebrally or intranasally. Infected lung tissue had an equally high titer. Owing to the high infectivity by intranasal inoculation, it seemed probable that the disease could be transmitted in mice by contact infection. But when on two occasions groups of normal mice were put into the cage with inoculated mice, the normal mice did not develop the disease; yet all of them were found to be fully susceptible when subsequently tested. An intranasal passage study was initiated from the ferret spleen material. The mice consistently developed extensive pneumonia. Another intranasal passage study was derived from a strain that had been through fifteen intracerebral passages. This strain failed to produce significant pulmonary lesions even after ten serial passages by intranasal inoculation. Despite the absence of appreciable pulmonary lesions, a fatal infection ensued within four days after inoculation. In both passage studies the lung tissue used for the inoculum was taken from mice killed seventy-two hours after injection. Mice were also tested for their susceptibility to infection through the skin. We were unable to infect the mice when a swab of cotton dipped in a 10 per cent suspension of infected tissue was rubbed on the skin; however, if a light needle scratch preceded the application, the mice often developed tularemia. Mice infected by various methods of inoculation consistently developed a *B. tularense* bacteremia.

TULAREMIA IN BABY CHICKS

Twelve 1-day-old Rhode Island Red chicks were injected intracerebrally with 0.03 c.c. of a 10 per cent suspension of the ferret spleen material. Two were prostrate on the third day. All were dead by the seventh day after inoculation. Groups of six chicks were inoculated with serial tenfold dilutions of

infected chick brain made up in distilled water. All of the chicks inoculated with the 10^{-4} dilution were dead by the fifth day, and 2 of the 6-chick groups inoculated with the 10^{-5} dilution developed tularemia.

TULAREMIA IN OTHER LABORATORY ANIMALS

Guinea pigs inoculated intracerebrally or intraperitoneally with suspensions of infected tissue were usually dead within four days. Normal guinea pigs failed to contract infection by contact with experimentally infected guinea pigs. Hamsters inoculated intracerebrally or intraperitoneally usually died on the third day after injection. Rabbits showed the same incubation period as guinea pigs and died regularly within four days after intracerebral or intraperitoneal inoculation. Ferrets inoculated intranasally with a 10 per cent suspension of infected mouse-lung tissue developed fever in about twenty-four hours, and were usually prostrate on the sixth day after injection.

CULTIVATION OF B. TULARENSE IN TISSUE CULTURE

Bacterium tularense multiplied readily in a medium of minced 9-day-old chick embryo tissue and serum Tyrode solution. Transfers were made after three to four days' incubation. The organism remained highly virulent for mice through thirty-one serial subcultures.

EFFECT OF PHYSICAL AND CHEMICAL AGENTS ON BACTERIUM TULARENSE

In the course of the tissue-culture experiments we occasionally inoculated flasks containing 4 c.c. of human serum Tyrode solution to see whether the organism would remain alive without the presence of chick embryo tissue. Material from these flasks did not prove infectious for mice after four days' incubation at 37° C.

Infected brain stored frozen at –25° C. retained its infectivity for several months as did also 10 per cent suspensions of infected tissue when they were tubed, sealed, and stored at –70° C. Repeated freezing and thawing of suspensions of infected tissue rendered the material avirulent.

Since dogs developed a fatal infection following intracerebral inoculation, we tested the effect of sulfadiazine in preventing a fatal outcome. Four dogs inoculated intracerebrally with 0.5 c.c. of a 1:1000 dilution of infected mouse brain were given 3 Gm. of sulfadiazine on the day of inoculation and then 1.5 Gm. daily. In this dosage the drug failed to show any inhibitory effect on the course of the disease. Four control dogs were tested in parallel with the treated dogs.

Various dilutions of infected tissue were passed through filters designed to withhold bacteria. The Seitz serum sterilizing filter pad was impervious to the organism. The infecting agent passed through the Berkefeld V grade filter quite readily, but was withheld by the Berkefeld N and W filters.

DISCUSSION

In view of the possible contamination of canine-distemper-virus material with viruses or bacteria not revealed by ordinary sterility tests, such products should not be released for sale until each lot of virus has been tested by animal inoculation and by culture on blood dextrose cystine agar. Guinea pigs or mice

can be used for the test inoculation; injection should be made intracerebrally.

Dogs and cats undoubtedly can be infected with tularemia in nature from eating infected rabbits or rodents. The question arises whether dogs can be infected by the bite of ticks carrying *B. tularense*. We have found that stray dogs apprehended in Montgomery County, Alabama, frequently show a 1:40 to a 1:80 agglutination titer with *B. tularense* antigen. The fact that the organism may persist for some time in the nasopharynx of infected dogs offers a possible means of conveying the infection to man. It remains to be proved whether ticks can become infected from feeding on dogs having tularemia.

The symptoms exhibited by dogs experimentally infected with tularemia are quite similar to those of canine distemper. The skin lesions are of the same type and distribution. Both diseases cause a primary febrile reaction associated with watery lacrimal and nasal discharge, and a secondary febrile period characterized by mucopurulent lacrimal and nasal discharge. This may be the reason why tularemia infections have not been recognized as such in dogs.

The epidemiology of tularemia in nature needs further investigation. Our limited study of contact infection was negative. This result was surprising since the animals are known to be susceptible to intranasal inoculation and since the organism is known to be present in the secretions of the respiratory tract. The question arises whether tularemia spreads among rabbits by contact infection. The rabbit tick seems to play the principal role in disseminating the disease in this species. Some animals, especially rodents, are apt to eat their dead, and this is a ready source of infection. Pollution of impounded waters and sluggish streams by animals dead of tularemia has been the source of widespread epizootics among wild animals.[7, 8, 9]

We were unable to demonstrate any neutralizing effect in vitro of tularemia immune serum. This is in agreement with the studies of Francis.[10] The fact that laboratory animals can be immunized with formalinized tissue vaccine raises the question whether a tissue vaccine might not be more efficacious than one prepared from a pure culture of *B. tularense*.

White mice are highly susceptible to tularemia and should prove valuable as test animals for the diagnosis of human infections. The early diagnosis of tularemia depends on the isolation of the organism, as the agglutination reaction does not become positive until the second to third week of the disease. Culture of blood on glucose cystine agar slants may be done, but intraperitoneal injection of whole blood into mice or guinea pigs is a more certain method of demonstrating a septicemia. The duration of infectivity in cases of tularemic pneumonia or tularemic nasopharyngitis can be determined by mouse inoculation. The occasional occurrence of tularemic pneumonia in man with no evident portal of entry and no history of contact with rabbits warrants attention to the possibility of infection from dogs.

The uniform fatality produced in dogs by intracerebral inoculation with *B. tularense* offers a means of studying the therapeutic effect of various chemicals and antisera on tularemic infections. The affinity of *B. tularense* for the central nervous system of mice and dogs is of interest, as tularemic meningitis has been reported in man.[1]

It must be emphasized that the identity of *B. tularense*-like organisms isolated by blood culture and animal inoculation must be substantiated by ag-

glutination tests. We have encountered an unidentified organism in blood cultures of dogs which closely resembles *B. tularense* in morphology and cultural characteristics. It is not antigenically related either to *B. tularense* or *Brucella bronchisepticus.*

SUMMARY

The isolation of *B. tularense* from dog spleen tissue used for the production of canine distemper vaccine is described. Attention is called to the danger of spreading lymphocytic choriomeningitis and tularemia through the medium of contaminated live virus-canine-distemper vaccine prepared from dog spleen.

Dogs and cats regularly develop a systemic infection following inoculation with a suspension of tissue infected with *B. tularense.* The organism shows a special affinity for the upper respiratory tract in both species. Puppies are more susceptible than mature dogs. Mature dogs, injected intracerebrally, develop a fulminating meningoencephalitis with a 100 per cent fatality. *B. tularense* was found to persist for as long as twenty-five days in the nasopharynx of dogs infected by intranasal inoculation. The organism was also recovered from the nasal secretion of dogs infected by feeding infected animal tissue, and from the lungs of dogs and cats inoculated subcutaneously.

Baby chicks are susceptible to infection with *B. tularense,* and the organism grows readily in a tissue culture medium of chick embryo tissue and serum Tyrode's solution.

A practical method of isolating *B. tularense* from sputum and nasopharyngeal secretions by mouse inoculation is described.

The author wishes to express his appreciation to Mrs. L. G. Johnson of the Alabama State Health Department Diagnostic Laboratory for performing the agglutination tests in this study.

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Vi AGGLUTINATIVE PROPERTIES FOR BACTERIUM TYPHOSUM DEMONSTRATED FOLLOWING INFECTION WITH MALARIA PARASITES

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SINCE the original report from this laboratory on the occurrence of Vi agglutination with *Bacterium typhosum* in sera from patients having therapeutic malaria,[1] specimens have been examined from those having clinical malaria and also from patients having neurosyphilis and treated by intravenous injection of typhoid vaccine. The only report found in the literature of the demonstration of Vi agglutination in a significant number of sera from individuals having infections with agents other than typhoid bacilli is that of Bensted.[2] He observed such reactions in specimens from six patients having tropical typhus fever, who had previously been inoculated subcutaneously with heat-killed typhoid vaccine. He obtained no Vi agglutination in sera from forty-four other similarly inoculated patients who had tonsillitis, bronchial catarrh, or tuberculosis. He demonstrated transient Vi agglutinative properties within a few days after subcutaneous administration of heat-killed phenolized typhoid vaccine, and suggested that latent or residual Vi agglutinative properties thus induced may subsequently be stimulated by other agents. Felix, Rainsford, and Stokes[3] and also Climie[4] demonstrated Vi agglutination in sera of individuals inoculated subcutaneously with alcohol-killed typhoid vaccine, but in sera from only a few when the vaccine was heat-killed and preserved with phenol. When heat-killed vaccine was administered intravenously, however, Climie observed Vi agglutination in a high percentage of cases.

The association of Vi agglutination with infection with malaria parasites was first suggested, in this laboratory, by results obtained with sera submitted in 1942 from inmates of Sing Sing Prison to be tested for floccular and granular agglutination with typhoid bacilli. As a matter of interest, the sera were also tested for Vi agglutination and, surprisingly, this occurred in relatively high dilutions of the specimens from three individuals who, it was learned later, had received subcutaneous injections of head-killed typhoid vaccine preceding malarial therapy. A systematic study was then undertaken of specimens from this group of patients, using sera remaining after tests for evidence of syphilis had been completed.

The technic outlined in a previous publication[5] was followed, and a pure Vi strain of *Bact. typhosum* isolated in this laboratory (No. 37136) was used, in addition to that designated by Bhatnagar[6] as "Vi I" (No. 39121). No significant difference was observed in the degree of reaction with the two strains. (Since the completion of this study, strain No. 37136 has become

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unsatisfactory for demonstrating Vi agglutination.) Tests for floccular and granular agglutination were performed as outlined in the Standard Methods[7] of this Division.

SERA FROM PATIENTS HAVING THERAPEUTIC MALARIA

Sera from thirty-four patients with neurosyphilis or latent syphilis who had been subjected to malarial therapy were studied. Definite Vi agglutination occurred in specimens from twenty-three, and partial or questionable reactions, in those from two. The failure to demonstrate Vi agglutination in sera from the remaining nine patients does not prove that these properties may not have been present at some time. Only one specimen was tested from each of six of the latter; blood examined from the other three was collected either within three weeks or not until four months or more after malarial therapy. Agglutinative properties may have been present in the intervening period since the examination of series of sera from ten other neurosyphilitic individuals indicates that reactions may not be demonstrated until the fifth or sixth week and may not persist for longer than three months after therapy. The peak usually occurred between the third and the fifth week.

Eight of these patients whose sera contained Vi agglutinative properties were said to have had typhoid vaccine: four, subcutaneously within a month; one, subcutaneously twenty-four years previously; and three, intravenously ten days, eighteen months, and twenty months, respectively, before the specimens were collected. On the other hand, fifteen whose sera reacted were reported not to have had typhoid vaccine after admission to Sing Sing, and to have stated that they had not had it previously. Significant floccular agglutination with typhoid bacilli occurred in sera from only one of the latter, suggesting that he may have had vaccine but had forgotten or failed to comprehend the meaning of the inoculation.

It is of particular interest that two attempts to infect one patient with malaria parasites were reported unsuccessful; yet serum collected six weeks later had a Vi agglutination titer of 1:20.

As a control, tests for Vi agglutination were performed with 103 sera from ninety-six inmates of Sing Sing Prison who had received no malarial therapy. Definite reactions were obtained in specimens from only two, in a 1:40 dilution of one and in a 1:10 dilution of the other. Partial reactions of questionable significance occurred in those from seven. The data indicated that typhoid vaccine had not been given to any of the nine. Blood specimens were secured a year later from the two whose sera gave definite reactions, and a titer of 1:40 was again obtained with one but no significant agglutination with the other. Series of three fecal specimens collected at three-day intervals from both individuals were examined for typhoid bacilli; none was found. This incidence of unexplained reactions is not unusual.[5]

SERA FROM PATIENTS HAVING CLINICAL MALARIA

In view of the incidence of Vi agglutination in sera from patients undergoing malarial therapy, specimens were obtained from twenty-eight men in the armed forces who had clinical malaria. Vi agglutinative properties were dem-

onstrated in sera from eleven of the twenty-eight. In four instances, Vi agglutination was not obtained in the first specimen but was demonstrated in those secured later. Only one specimen was received from each of twelve of seventeen men whose sera failed to react. The date of onset in five of the latter was said to be several months prior to the collection of blood, and in one, only two days; in the remaining eleven, no information regarding the duration or stage of the disease was furnished, and thus the findings cannot be evaluated. Although no information regarding typhoid vaccine was supplied, it may be assumed that all had had prophylactic inoculations.

SERA COLLECTED AFTER VACCINE THERAPY

For comparison, and in view of reports of Bensted and Climie, sera from patients having neurosyphilis treated by the intravenous injection of heat-killed phenolized typhoid vaccine were tested. The specimens were submitted for serologic tests for syphilis in the control of treatment of patients in Bellevue Hospital, New York. Specimens from fourteen of thirty-one individuals gave definite Vi agglutination. The reaction usually occurred within a week after vaccine therapy and persisted for only a short time.

Table I shows the findings in representative cases from the different series. The results of Vi agglutination tests in relation to clinical and therapeutic data are summarized in Table II, and in relation to granular and floccular agglutination with the typhoid bacillus, in Table III. The latter indicates

TABLE I

TITER OF VI AGGLUTINATION IN REPRESENTATIVE SERA

CLINICAL AND THERAPEUTIC DATA	SPECIMEN COLLECTED	AGGLUTINATION TITER WITH TYPHOID BACILLI		
		Vi	GRANULAR	FLOCCULAR
Patient No. 1. Neurosyphilis.	9/18/42	1:160	–	–
Malarial therapy 8/18/42.	10/ 5/42	1:80	–	–
No history of typhoid vaccine.	10/14/42	1:80	–	–
	11/ 9/42	1:10	–	–
	11/30/42	–	–	–
Patient No. 2. Neurosyphilis.	8/ 5/42	1:10	1:80	1:320
Malarial therapy 8/5/42.	8/10/42	1:20	1:80	1:320
Typhoid vaccine subcutaneously in 1918.	9/21/42	1:160	1:20	1:80
	10/ 5/42	1:80	1:10	1:160
	11/ 9/42	1:10	1:20	1:160
	12/ 3/42	1:10	–	1:80
Patient No. 3. Clinical malaria.	3/25/43	1:80	–	1:80
Onset 3/22/43.	3/30/43	1:640	–	1:160
Patient No. 4. Clinical malaria.	3/18/43	–	–	1:80
Onset 3/16/43.	3/20/43	–	1:20	1:160
Patient No. 5. Clinical malaria.	2/24/43	–	–	1:640
No information furnished concerning	3/ 2/43	1:80	–	1:2500
date of onset.	3/ 9/43	1:40	–	1:1200
Patient No. 6. Neurosyphilis.	3/16/43	–	–	1:20
Typhoid vaccine intravenously begun	3/29/43	1:80	1:2500	1:20,000
3/16/43.	4/30/43	1:40	1:1200	1:1200
	6/14/43	1:10	1:80	1:160
Patient No. 7. Neurosyphilis.	3/19/43	–	–	–
Typhoid vaccine intravenously begun	3/30/43	1:20	1:160	1:2500
3/19/43.	4/27/43	–	1:1200	1:1200
Patient No. 8. Neurosyphilis.	3/26/43	–	–	–
Typhoid vaccine intravenously begun	4/ 6/43	–	1:320	1:10,000
3/26/43.	5/ 4/43	–	1:40	1:320

that the incidence and degree of Vi agglutination reactions do not appear to be directly related to floccular or granular agglutination of typhoid bacilli. Neither do they appear to be related in any way to the complement-fixation reactions in the tests for evidence of syphilis.

TABLE II

SUMMARY OF Vi AGGLUTINATION REACTIONS IN RELATION TO MALARIA AND TYPHOID VACCINE

HISTORY OF MALARIA	HISTORY OF TYPHOID VACCINE	NUMBER OF INDIVIDUALS WHOSE SERA GAVE	
		DEFINITE Vi AGG.	NO SIGNIFICANT Vi AGG.
Therapeutic malaria	Subcutaneous	5	1
	Intravenous	3	0
	No history of vaccine	15	10
Clinical malaria	Subcutaneous assumed	11	17
No malaria	Intravenous	14	17
	No history of vaccine	2	7
	Not determined	0	87

TABLE III

INCIDENCE OF Vi AGGLUTINATION IN RELATION TO GRANULAR AND FLOCCULAR AGGLUTINATION WITH TYPHOID BACILLI

DEGREE OF Vi AGGLUTINATION	NUMBER OF SERA IN WHICH GRANULAR OR FLOCCULAR AGGLUTINATION OCCURRED IN					
	NO SIGNIFICANT DILUTION	DILUTIONS OF				TOTALS
		1:40 or 1:80	1:160 or 1:320	1:640 or 1:1200	1:2500 or greater	
None	100	50	37	17	18	222
Partial	12	6	6	0	2	26
Definite	32	14	22	12	14	94

DISCUSSION

One hypothesis for the frequent demonstration of Vi agglutinative properties following infection with malaria parasites is that the plasmodia or their products may possess an antigenic property previously demonstrated only in typhoid and closely related bacilli. A second explanation may be the occurrence of an anamnestic reaction; that is, stimulation of latent agglutinative properties by a nonspecific antigen, it being assumed that such antibodies were previously produced by microorganisms containing Vi antigen.

Bensted's findings indicate that transitory Vi agglutinative properties may be induced by subcutaneous injection of heat-killed phenolized typhoid vaccine, and that these properties may reappear later as a result of stimulation by a nonspecific antigen. No explanation appears, however, for the reactions in sera from individuals who gave no history of having had typhoid vaccine. It is possible that in these instances, the latent agglutinative properties had been produced by salmonella or members of the coliform group that have been shown to possess Vi antigen. A systematic study of other bacterial species might furnish significant information. From an academic standpoint, the observations present a new problem. They are of practical importance, since a new limitation is placed on a reaction generally considered of specific epidemiologic significance.

SUMMARY

Vi agglutinative properties were demonstrated with typhoid bacilli in significant dilutions of sera from (1) twenty-three of thirty-four patients having neurosyphilis or latent syphilis and who were receiving malarial therapy; (2) two of ninety-six individuals who had received no malarial therapy; (3) eleven of twenty-eight patients with clinical malaria; and (4) fourteen of thirty-one neurosyphilitic patients who had been treated by intravenous injection of typhoid vaccine.

Two hypotheses are offered: (1) that malaria parasites possess Vi antigen and (2) that latent agglutinative properties are stimulated.

These observations should be considered in evaluating Vi agglutination. A continuation of the study should include the examination of various species of bacteria for Vi antigen and the determination of factors that may stimulate latent Vi agglutinative properties.

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STUDIES ON THE ABSORPTION OF THE SULFONAMIDES FROM THE GASTROINTESTINAL TRACT OF ALBINO RATS

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MANY reports on the pharmacology of the sulfonamides in human beings and animals have been published.[1] In these studies, with few exceptions,[2] observations on the absorption of the sulfonamides as determined by blood levels obtained were seldom made before one hour after their administration to human beings by mouth or by stomach.

Recently, we reported the results of investigations in human beings[3] in which we observed the blood levels reached by various sulfonamides during a three-hour period after their administration by mouth. We found that (1) sulfapyridine, sulfathiazole, and sulfadiazine, as well as their sodium salts, were quickly absorbed from the gastrointestinal tract, as evidenced by their rapid appearance in the blood, frequently as early as five minutes after peroral administration; (2) the absorption of sulfapyridine, sulfathiazole, and sulfadiazine was favored by the simultaneous administration of equivalent amounts of sodium bicarbonate; (3) the absorption of the sodium salts, as demonstrated by blood levels attained, was more rapid and greater than that obtained when sulfapyridine, sulfathiazole, or sulfadiazine were given alone or in combination with sodium bicarbonate; (4) conjugated forms of the sulfonamides appeared in the blood as early as five minutes after administration, in some instances being the only forms present; and (5) conjugation of the sulfonamides was greater when the sodium salts were administered than when sulfapyridine, sulfathiazole, and sulfadiazine with bicarbonate of soda were given.

It has been the opinion of some workers in the field of chemotherapy[4] that absorption of the sulfonamide compounds occurred principally from the small intestine. This concept was based on investigations made after the administration of sulfapyridine by stomach tube to dogs in which the pylorus had been ligated; insignificant amounts of the sulfonamide were found to be absorbed from the stomach. Similar doses administered into the small intestine produced significantly higher blood levels. This study was confined to the levels of free drug in the blood, since conjugation of the sulfonamides was found to occur only slightly in dogs.

In view of our studies showing the rapid absorption of the sulfonamides as indicated by their early appearance in the blood after peroral administration in human beings, we felt that, despite reports to the contrary,[4] significant quantities of these drugs could be absorbed from the stomach. The purpose of the

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present study is to investigate in albino rats the absorption of the sulfonamides from the stomach, as well as to compare these results with those from the stomach and small intestine together.

METHODS AND MATERIALS

The animals used were healthy albino rats, each weighing from 300 to 350 grams. The animals in one group were anesthetized lightly with ether. The abdominal wall was incised, the stomach exposed, the pylorus occluded by a black silk ligature, and the abdomen closed with metal skin clips. Administration of the sulfonamides by gavage was accomplished, and the animals were permitted to return to consciousness within five minutes after completion of the operative procedure.

A second group of animals, in which pyloric occlusion was not done, were given the sulfonamides also by gavage.

Sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine were administered in doses of ⅓ Gm. of the sulfonamide per kilogram of body weight. This dosage was chosen arbitrarily, the amount of the drug being proportionately the same as that which Long in studies on sulfonamide absorption, administered to mice.[5] It also was the daily amount of sulfonamide administered by us in the treatment of pneumococcal pneumonia in rats.[6]

Samples of blood for determination of its sulfonamide content were obtained by cardiac puncture five minutes after administration of the drug, and then at intervals of five and ten minutes during the first half-hour and second half-hour periods, respectively. From six animals with pyloric occlusion, to each of which was administered a different sulfonamide, specimens of blood were obtained at intervals of one minute during the first five minutes after administration.

Determinations of free and conjugated sulfonamides in the blood were made according to the procedure of Bratton and Marshall.[7] A Klett-Summerson photoelectric colorimeter with a No. 54 filter was used.

RESULTS

Sulfapyridine (Charts 1 and 2).—Small amounts of free sulfapyridine (0.072, 1.17, 1.36, and 2.29 mg. per 100 c.c.), were found in the blood in each of four rats with pyloric occlusion five minutes after sulfapyridine had been given. In each of three animals without pyloric occlusion, levels of 1.6, 6.5, and 7.6 mg. per 100 c.c. of the free drug were found.

In three of the four animals with pyloric occlusion both the free and conjugated forms were found in the blood at five minutes after administration, while in three rats without pyloric occlusion the conjugated form was found in one.

In the animals without pyloric occlusion, it was found that the blood levels in two of the three were higher throughout the hour period than in those with pyloric occlusion. In a third animal without pyloric occlusion, which survived only twenty-five minutes, the levels of free drug in the blood were similar to

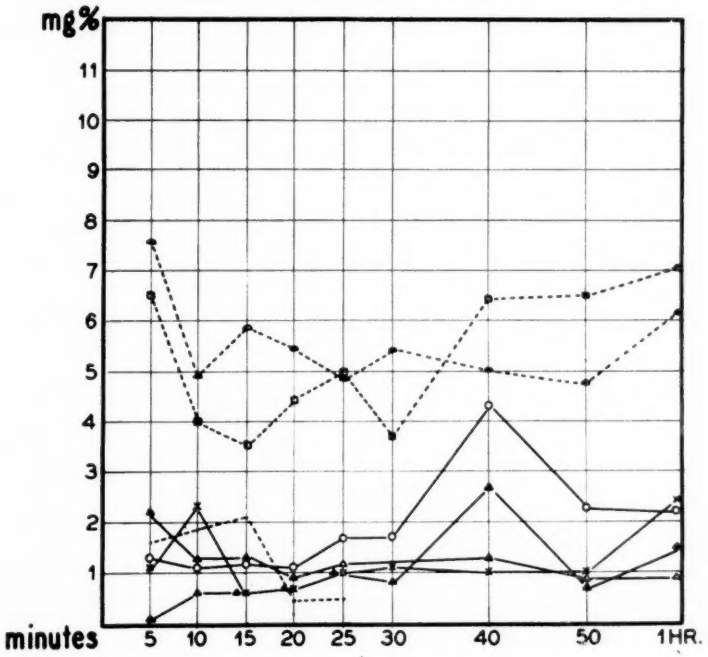

Chart 1.—Free blood levels of sulfapyridine after a single dose of ⅓ Gm. per kilogram of sulfapyridine administered by gavage.

---- Without pyloric occlusion.
—— With pyloric occlusion.

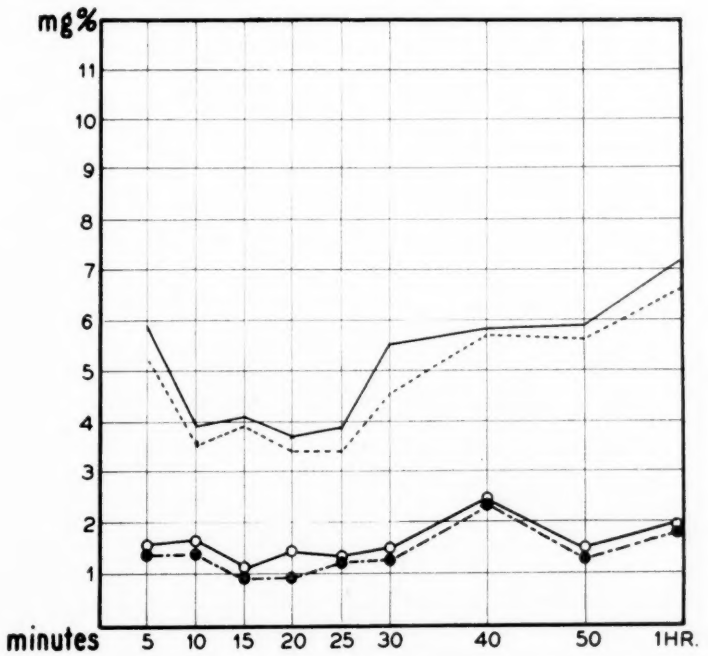

Chart 2.—Average free and total blood levels of sulfapyridine after a single dose of ⅓ Gm. per kilogram of sulfapyridine administered by gavage.

---- Total
—— Free Without pyloric occlusion.

o—o Total
•---• Free With pyloric occlusion,

those in the animals with pyloric occlusion. The degree of conjugation in each, however, was approximately the same.

In two animals with pyloric occlusion to which sulfapyridine had been administered, determinations of the blood sulfonamide were made at one-minute intervals during the first five-minute period. In one animal, free levels of 12.0, 7.7, 7.6, 5.76, and 4.65 mg. per 100 c.c., respectively, were obtained at the first,

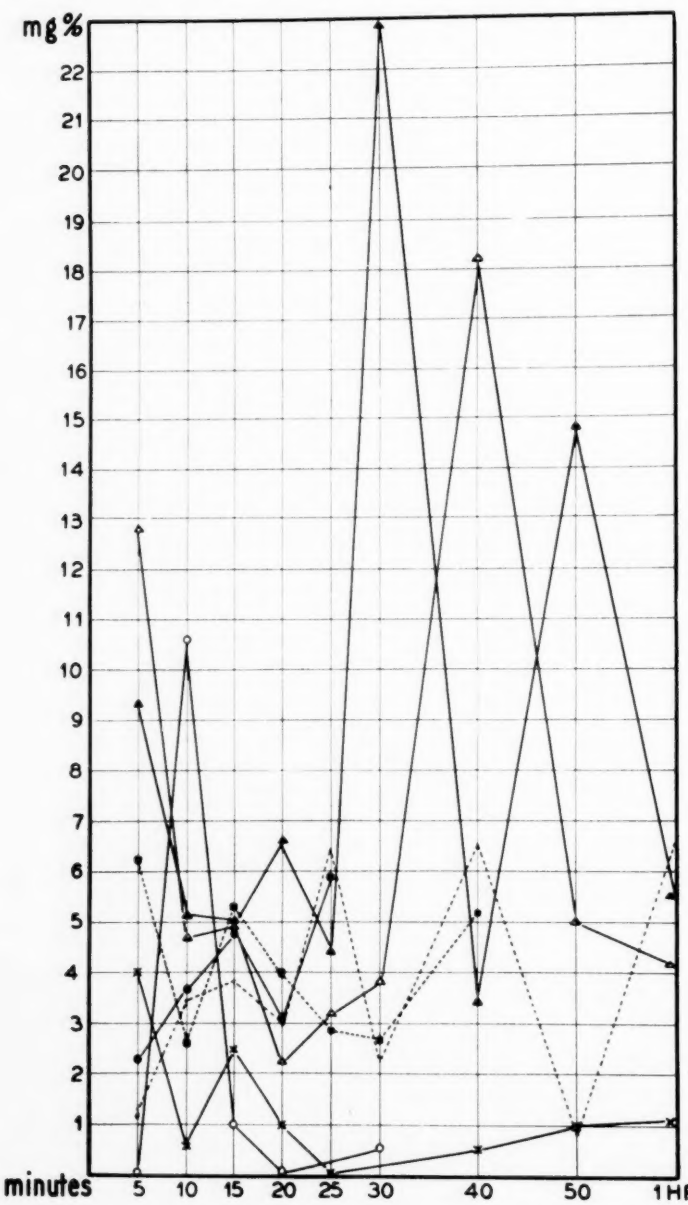

Chart 3.—Free blood levels of sodium sulfapyridine after a single dose of ⅓ Gm. per kilogram of sodium sulfapyridine administered by gavage.

---- Without pyloric occlusion.
—— With pyloric occlusion.

second, third, fourth, and fifth minutes. Total levels of the drug in this animal were respectively 12.0, 8.0, 7.6, 5.97, and 4.97 mg. per 100 c.c. A second animal similarly treated had free blood levels of 6.13, 5.33, 3.4, and 2.0 mg. per 100 c.c., and total blood levels of 8.26, 6.93, 4.53, and 4.13 mg. per 100 c.c. at one, two, three, and five minutes.*

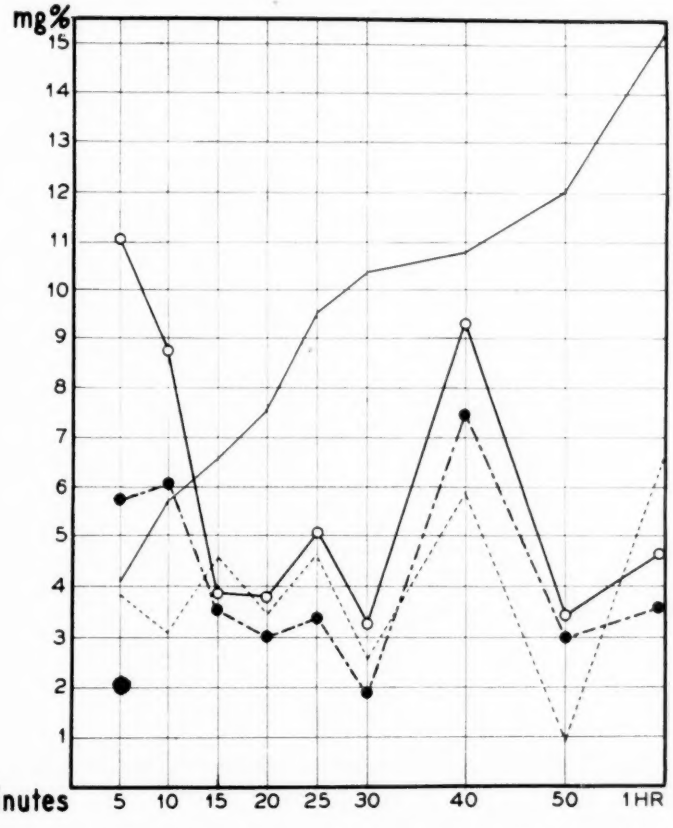

Chart 4.—Average free and total blood levels of sodium sulfapyridine administered by gavage.

---- Total
—— Free Without pyloric occlusion.

o—o Total
•--• Free With pyloric occlusion.

Sodium Sulfapyridine (Charts 3 and 4).—Following the administration of sodium sulfapyridine, greater quantities of sulfonamide appeared in the blood of each group of animals at five minutes than when sulfapyridine was given. In the rats with pyloric occlusion, the free levels of sodium sulfapyridine in the blood were 0.1, 2.3, 4.0, 9.3, and 12.9 mg. per 100 c.c. and without pyloric occlusion the free levels were 1.4 and 6.3 mg. per 100 c.c.

In three of five animals with pyloric occlusion to which sodium sulfapyridine had been administered, the conjugated as well as the free form was present at five minutes. In a fourth rat the conjugated form alone was present. In two rats without pyloric occlusion, one had conjugated as well as free sodium sulfapyridine in the blood.

*Blood could not be obtained at the fourth minute.

In one animal with pyloric occlusion to which sodium sulfapyridine had been administered, blood sulfonamide determinations were made at minute intervals during the first five-minute period. The free levels obtained were 4.4 and 8.9 mg. per 100 c.c. at one and two minutes, respectively. Traces were found at three and four minutes, while at five minutes, the free form had disappeared entirely, though the conjugated form was present. Conjugated sulfapyridine which appeared as early as one minute after administration, was absent at the second minute, reappeared at the third, and was present at the fourth and fifth minutes.

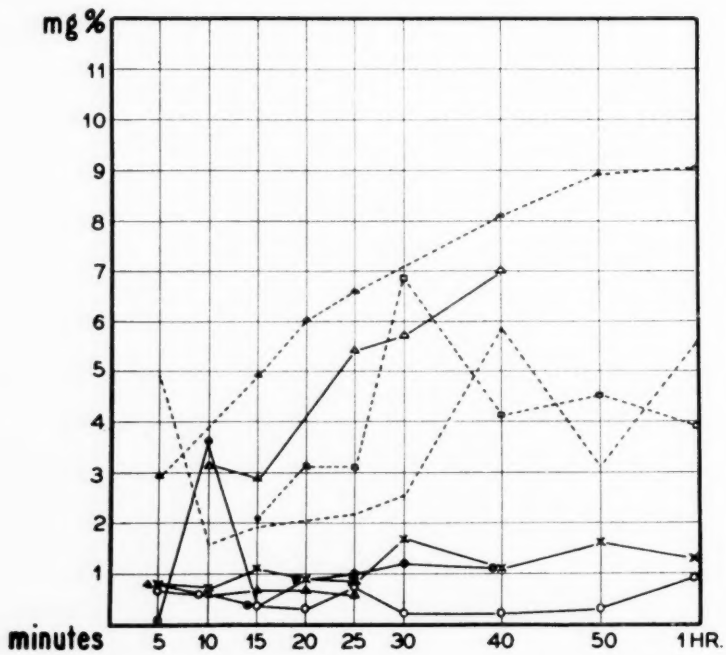

Chart 5.—Free blood levels of sulfathiazole after a single dose of ⅓ Gm. per kilogram of sulfathiazole administered by gavage.
---- Without pyloric occlusion.
—— With pyloric occlusion.

In the rats receiving sodium sulfapyridine, the free blood levels in those with pyloric occlusion were frequently higher during the hour period than in those without pyloric occlusion. In both groups wide fluctuations occurred, being more pronounced in those with pyloric occlusion. Whereas, the average total level generally ranged close to the free level in those animals whose pylorus was occluded, in those without occlusion the average degree of conjugation progressively increased throughout the entire hour.

Sulfathiazole (Charts 5 and 6).—Free sulfathiazole in small amounts (0.84, 0.84, 0.78, and 0.12 mg. per 100 c.c.), was found in the blood in each of four animals with pyloric occlusion five minutes after the administration of sulfathiazole,* while, in each of two animals without pyloric occlusion, significantly larger quantities (2.9 and 4.8 mg. per 100 c.c.) were present.

*Blood could not be obtained from a fifth rat at five minutes.

In all four rats with pyloric occlusion, conjugated sulfathiazole was present in addition to the free form at five minutes, and one of the two rats without pyloric occlusion had both conjugated and free drug in the blood.

In one rat with pyloric occlusion, determinations for the sulfonamide in the blood at intervals of one minute for the first five minutes were made. Free blood levels in this animal were 0.84, 0.72, 1.15, and 0.84 mg. per 100 c.c. respectively at one, two, three, and five minutes after administration. No specimens of the blood were procured at the fourth minute. Total blood levels, revealing the presence of conjugated sulfathiazole were 1.2, 0.78, and 1.2 mg. per 100 c.c., respectively, at one, two, and five minutes. No determination for conjugated drug was made at three minutes.

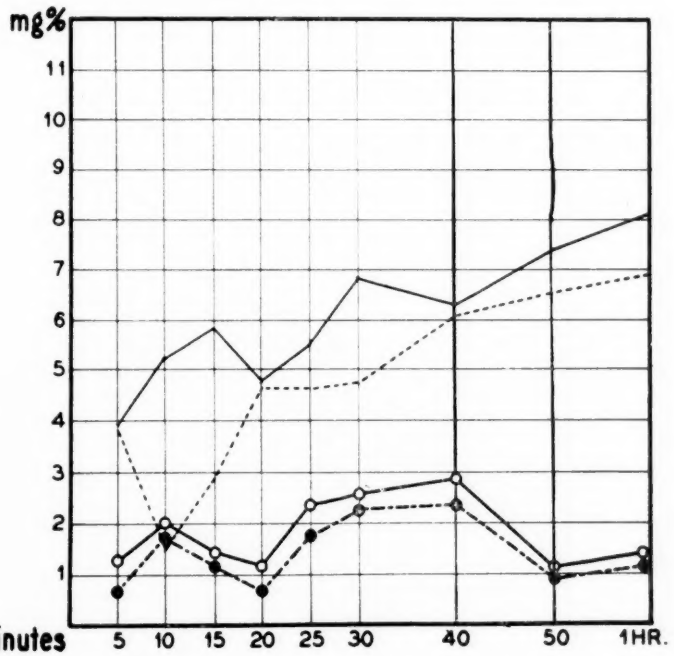

Chart 6.—Average free and total blood levels of sulfathiazole after a single dose of ⅓ Gm. per kilogram of sulfathiazole administered by gavage.

---- Total
—— Free Without pyloric occlusion.

Among those animals with pyloric occlusion to which sulfathiazole was administered, four had free blood levels continuing until death below 2.0 mg. per 100 c.c. with but one exception, a temporary rise to 3.6 mg. per 100 c.c. at ten minutes. A fifth animal presented a continued rise in the blood level until death at forty minutes, at which time it had reached 7.0 mg. per 100 c.c. The three animals without pyloric occlusion had free blood levels ranging from 1.6 mg. per 100 c.c. at ten minutes, to 9.0 mg. per 100 c.c. at one hour. In two of these animals, the blood levels fluctuated, while the third had a steady rise to 9.0 mg. per 100 c.c. until death at sixty minutes.

Sodium Sulfathiazole (Charts 7 and 8).—In those animals to which sodium sulfathiazole had been given and in which pyloric occlusion had been done, blood levels of free sulfathiazole in each of four animals were respectively

3.2, 4.0, 4.7, and 7.3 mg. per 100 c.c. at five minutes after administration; whereas, in three without pyloric occlusion, the free levels were respectively 0.5, 3.2, and 8.8 mg. per 100 c.c.

In two of three animals with pyloric occlusion in which conjugated levels were determined at five minutes after administration of sodium sulfathiazole, there was conjugated as well as free sulfathiazole. In each of three determinations in those without pyloric occlusion both the free and conjugated forms were present.

Determinations of the blood sulfathiazole level in one animal with pyloric occlusion, at one minute, two minutes, and five minutes after administration of sodium sulfathiazole, were respectively 3.24, 3.50, and 3.10 mg. per 100 c.c. The

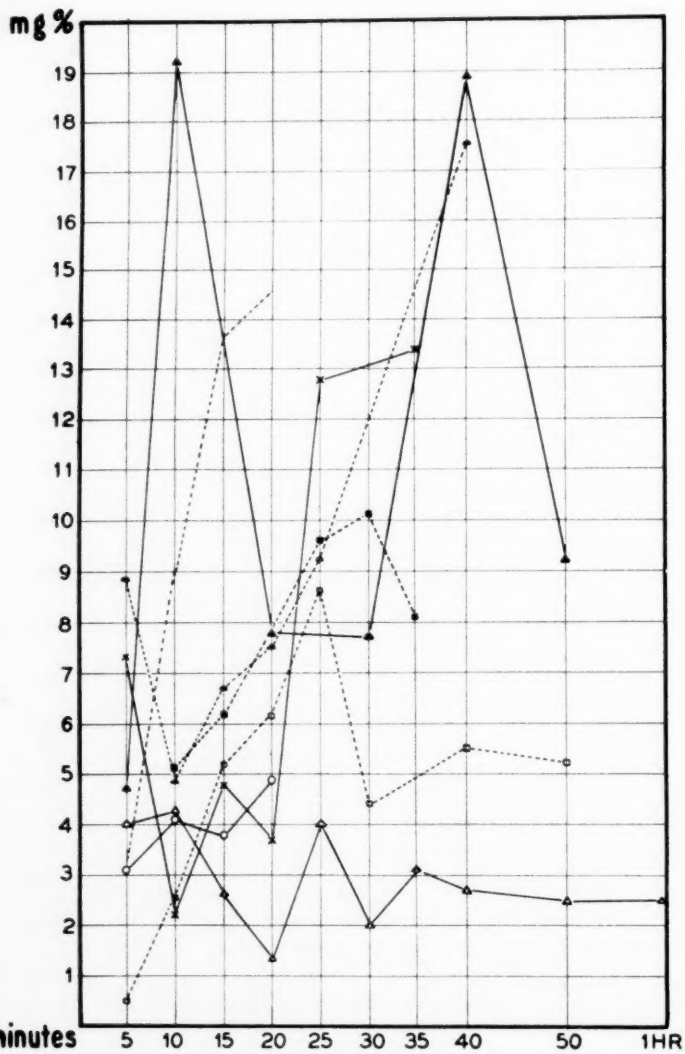

Chart 7.—Free blood levels of sodium sulfathiazole after a single dose of ⅓ Gm. per kilogram of sodium sulfathiazole administered by gavage.

---- Without pyloric occlusion.
—— With pyloric occlusion.

total drug at two minutes was 4.98 mg. per 100 c.c. Determinations of the total drug could not be made at one and five minutes.

In the animals to which sodium sulfathiazole had been administered, it was found that in those with pyloric occlusion, the levels of free drug in the blood, although they fluctuated widely, were similar to those obtained in those animals without pyloric occlusion. The amount of conjugated drug in those with

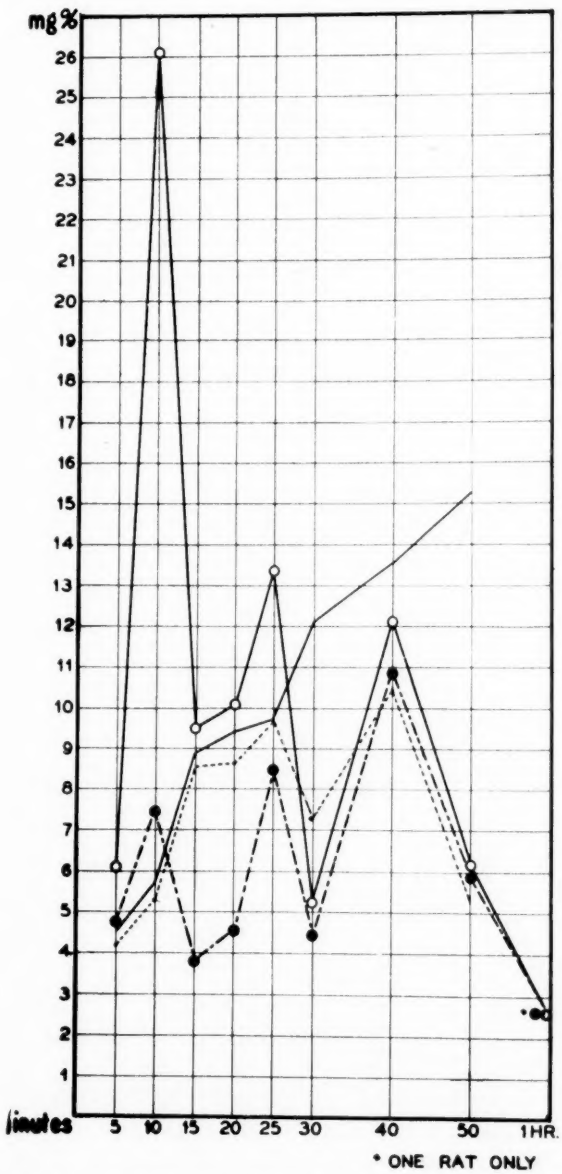

Chart 8.—Average free and total blood levels of sodium sulfathiazole after a single dose of ⅓ Gm. per kilogram of sodium sulfathiazole administered by gavage.

---- Total
—— Free Without pyloric occlusion.

o—o Total
•- -• Free With pyloric occlusion.

occlusion of the pylorus, however, while high at first, diminished rapidly, so that only a slight difference existed at thirty minutes and thereafter. In those without pyloric occlusion, the amount of conjugated drug increased progressively.

Sulfadiazine (Charts 9 and 10).—Sulfadiazine was present in the blood (0.9, 1.3, 1.5, and 1.5 mg. per 100 c.c.) in all four rats with pyloric occlusion five minutes after it had been given, and in both rats without pyloric occlusion (0.5 and 1.5 mg. per 100 c.c.).*

Three of the four rats without pyloric occlusion had conjugated as well as free sulfadiazine in the blood, and one of two without pyloric occlusion had conjugated drug at five minutes.

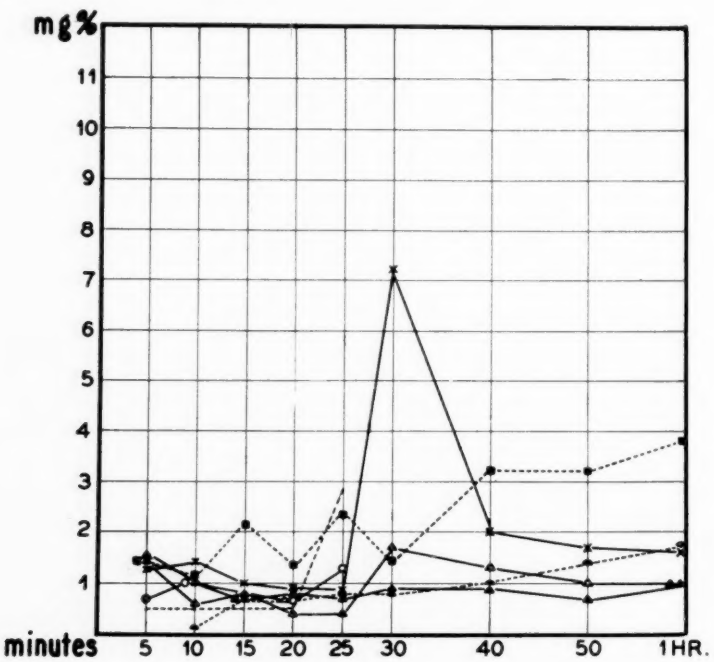

Chart 9.—Free blood levels of sulfadiazine after a single dose of ½ Gm. per kilogram of sulfadiazine administered by gavage.
---- Without pyloric occlusion.
—— With pyloric occlusion.

In one rat with pyloric occlusion determinations for sulfonamide in the blood were made at intervals of one minute during the first five minutes. These revealed free blood levels of 0.9, 0.6, 0.9, 0.8, and 1.5 mg. per 100 c.c., respectively, at one, two, three, four, and five minutes. Levels of conjugated drug found at the first, second, third, fourth, and fifth minute periods were 1.54, 1.46, 1.92, 1.73, and 2.32 mg. per 100 c.c.

In subsequent determinations made during the remainder of the hour, it was found that the levels in the four animals with pyloric occlusion were closely similar to those without pyloric occlusion during the first twenty-five minutes. After this time, in three of the animals with pyloric occlusion, two

*No blood could be obtained at five minutes from the third animal without pyloric occlusion.

of which survived for one hour, the levels were similar to those without occlusion. The third rat with pyloric occlusion showed a rapid rise in free level of sulfadiazine in the blood from 0.9 mg. per 100 c.c. at twenty-five minutes to 7.2 mg. at thirty minutes, and a subsequent fall to 2.0 mg. at forty minutes; thereafter, the levels in the blood coincided with the trend of the rest of the group. One of the two animals without pyloric occlusion had a gradual rise in free blood sulfadiazine from 1.5 mg. per 100 c.c. at thirty minutes to 3.4 mg. per 100 c.c. at one hour. The other attained free blood sulfadiazine levels similar to those found in the animals with pyloric occlusion.

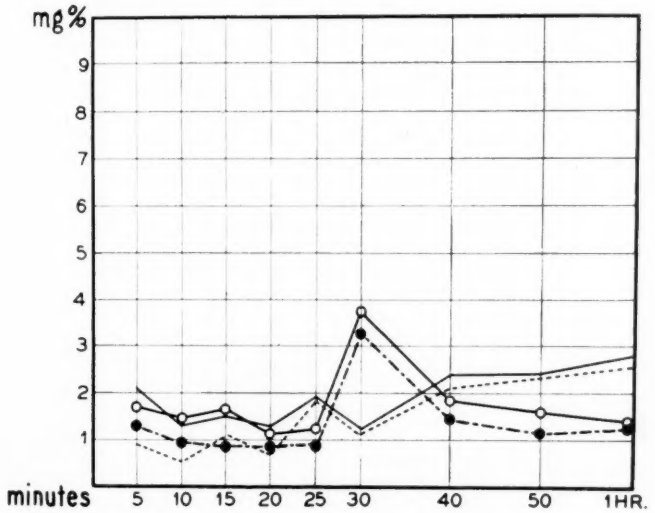

Chart 10.—Average free and total blood levels of sulfadiazine after a single dose of ⅓ Gm. per kilogram of sulfadiazine administered by gavage.

---- Free
—— Total Without pyloric occlusion.

o—o Free
•- -• Total With pyloric occlusion.

Sodium Sulfadiazine (Charts 11 and 12).—Following the administration of sodium sulfadiazine, all animals with pyloric occlusion had free sulfadiazine in the blood (0.3, 1.1, 1.3, 2.0, and 3.1 mg. per 100 c.c.) at five minutes. Very much greater levels were obtained in two animals without pyloric occlusion (10.6 and 13.1 mg. per 100 c.c.).*

Three animals with pyloric occlusion survived for fifty minutes or longer. Two had levels of 1.0 mg. per 100 c.c. or below. A third had levels between 2.5 and 6.0 mg. per 100 c.c. Two of the animals without pyloric occlusion had very high free levels. One had a progressive rise, which at first was sudden, from 13.0 mg. at five minutes to 27.2 mg. per 100 c.c. at fifty minutes. Another free sodium sulfadiazine level, after reaching 23.0 mg. per 100 c.c. at twenty minutes fell quickly to 7.8 mg. per 100 c.c. and then again rapidly rose to 15.4 mg. per 100 c.c. at the end of one hour. The free blood sulfadiazine level in a third animal initially was 10.6 mg. per 100 c.c. at five minutes, fell to 2.2 at twenty minutes, and 1.3 mg. at forty minutes; there then followed a gradual

*No blood was procured from a third animal at five minutes.

rise to 5.1 mg. per 100 c.c. at one hour. The degree of conjugation was slight in those animals with pyloric occlusion throughout the hour period. In those without pyloric occlusion, there was no conjugation during the first twenty minutes; however, during the next twenty minutes the average degree of conjugation increased chiefly because of lowered free levels, but from forty minutes to one hour there was less conjugation than during the previous period, with again slowly rising free levels which almost paralleled the total blood levels.

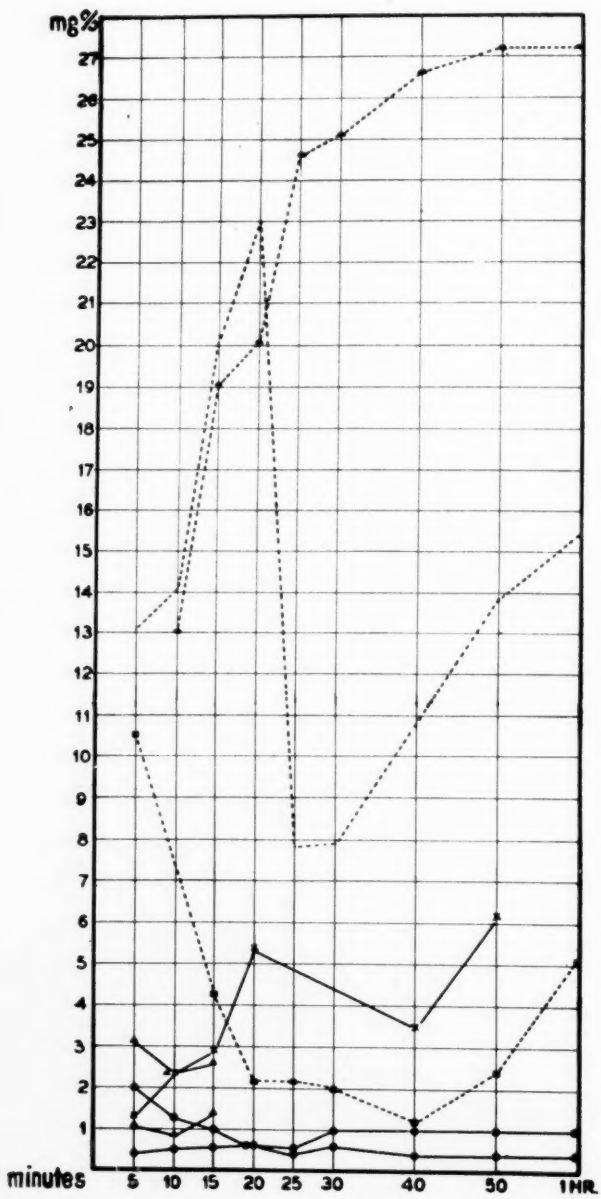

Chart 11.—Free blood levels of sodium sulfadiazine after a single dose of ⅓ Gm. per kilogram of sodium sulfadiazine administered by gavage.

---- Without pyloric occlusion.
—— With pyloric occlusion.

In another rat with pyloric occlusion to which sodium sulfadiazine had been given, free blood levels of 1.3, 1.4, and 1.2 mg. per 100 c.c. were obtained at one, four, and five minutes. Conjugated sulfadiazine, as well as free, was found at one and four minutes.

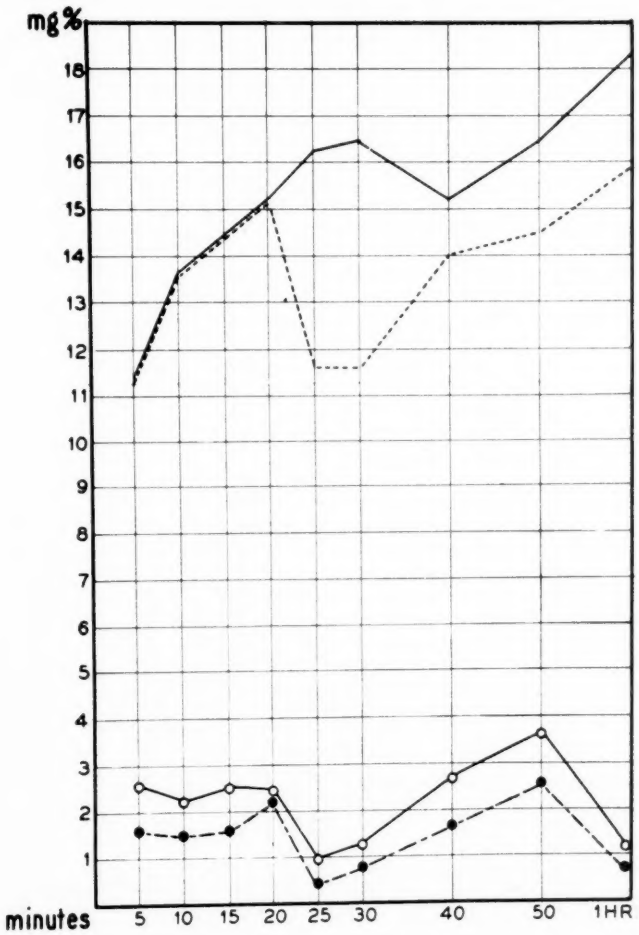

Chart 12.—Average free and total blood levels of sodium sulfadiazine after a single dose of ⅓ Gm. per kilogram of sodium sulfadiazine administered by gavage.

---- Total
—— Free Without pyloric occlusion.

o—o Total
•- -• Free With pyloric occlusion.

SUMMARY

Study was made of the absorption of sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine as evidenced by blood level determinations made at short intervals during a one-hour period following their administration by gavage to two groups of albino rats. In one group, the animals were normal; in the other, mechanical occlusion of the pylorus had just been effected.

Sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine were absorbed rapidly in both groups of

animals as shown by their appearance in the blood in all determinations made at five minutes after their administration by stomach tube.

Six animals in the group with pyloric obstruction, each of which had received a different drug, and in which blood samples were taken at minute intervals for five minutes after administration, had significant quantities of the sulfonamide in the blood as early as one minute.

Conjugated forms of the sulfonamides were present as early as one minute after administration in four of these six animals; in the other two, they appeared at two minutes.

The majority of the animals in both groups had conjugated sulfonamide in the blood five minutes after administration.

Greater amounts of sulfapyridine and sulfathiazole were found in the blood of those animals without pyloric occlusion than in those rats with pyloric occlusion. There was no essential difference between the free and total blood levels of sulfadiazine in those with or without pyloric occlusion.

The average degree of conjugation was greater in most instances in those animals to which sodium sulfapyridine, sodium sulfathiazole, and sodium sulfadiazine were administered, than in those receiving respectively, sulfapyridine, sulfathiazole, and sulfadiazine.

The free and total blood levels closely paralleled each other in those animals, which received sulfapyridine and sulfadiazine, with and without pyloric occlusion. In those animals with pyloric occlusion which received sulfathiazole, the free and total levels, likewise, closely paralleled each other, but in those without occlusion moderately wide divergences occurred at irregular intervals.

The rats given sodium sulfapyridine and sodium sulfathiazole responded similarly whether or not pyloric occlusion had been effected. Except for occasional fluctuations, there were no significant differences in the free blood levels of these two groups. In the rats with pyloric occlusion, the total levels paralleled the free levels with temporary exceptions. In the animals without occlusion which received sodium sulfapyridine, the free and total levels closely paralleled each other during the first twenty-five minutes; the same was true for sodium sulfathiazole during the first fifteen minutes. After this, the free and total levels with both sodium sulfapyridine and sodium sulfathiazole became widely divergent with continued increase of the conjugated form.

The animals without occlusion to which sodium sulfadiazine was given had very much higher blood levels than those rats with pyloric occlusion, the differences between free levels ranging up to 15 mg. per cent. The levels of the free and total sulfadiazine in the blood paralleled each other in the rats with pyloric occlusion, but remained approximately the same in those without occlusion for the first twenty minutes; after that, a wide divergence occurred.

CONCLUSIONS

1. Sulfapyridine, sodium sulfapyridine, sulfathiazole, sodium sulfathiazole, sulfadiazine, and sodium sulfadiazine seem to be absorbed from the stomachs of albino rats in significant amounts. With the exception of sulfadiazine, however, significantly greater quantities of the sulfonamides are absorbed from the gastrointestinal tracts of those animals without pyloric occlusion.

2. Conjugation of the sulfonamides in albino rats occurs as early as one minute after administration by gavage.

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COLD AGGLUTININS IN TUBERCULOSIS

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RECENT investigations of cold agglutinins in the blood serum have shown that their presence may be used as a diagnostic test for atypical pneumonias.[1, 2] This we have found to be true in a small number of cases which have been under supervision at this hospital. Still more recently, Siefert and Krautman[3] reported cold agglutinins present in the serum of patients with tuberculosis. This possibility was thought worthy of investigation because of difficulties which might arise if both tuberculosis and atypical pneumonia should exist at the same time in the same individual. The report of our investigation is as follows:

SELECTION OF CASES

Fifty individuals were used for the tests. The selection was made on the basis of activity and extent of disease. The group thus selected was classified as follows: active, minimal, 15; active, moderately advanced, 16; active, far advanced, 13; inactive, moderately advanced, 1; nontuberculous, 5.

DONORS

Cells from four Group 0 donors were used. Three of these donors were nontuberculous, and one was classified as active, minimal tuberculosis. The serum of each patient tested was used with the cells of more than one donor, and donors' cells were tested with their own sera as well as the sera of patients.

TECHNIQUE

Each patient's serum was diluted with physiological saline in twelve tubes from a 1:2 to a 1:4,096 dilution. Five-tenths cubic centimeter of a 2 per cent suspension of washed human Group 0 cells was then added, mixed with the diluted serum and placed in the refrigerator at 0° C. temperature overnight. Readings were taken immediately upon removal from the refrigerator and one hour later (in positive cases).

RESULTS

In no instance were we able to demonstrate cold agglutinins in any of the sera of patients with tuberculosis only. In two instances, however, where atypical pneumonia as well as tuberculosis was present, positive reactions were recorded.

Received for publication, May 24, 1944.

SUMMARY

In a series of fifty cases of tuberculosis the test for cold agglutinins was negative. This leads us to the opinion that tuberculosis in itself does not produce these agglutinins. We therefore conclude that the test has a distinct value in differentiating between tuberculosis and atypical pneumonia.

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THE INCIDENCE OF PLASMA-COAGULATING STAPHYLOCOCCI IN THE FECES OF CHRONIC INVALIDS

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IT IS possible[1] to detect plasma-coagulating staphylococci in feces by plating the sample on bromthymol blue lactose agar to which has been added potassium tellurite (either 0.10 c.c. of 0.10 per cent tellurite added to the surface of a previously poured plate or, better still, 0.10 c.c. of 5.0 per cent unheated tellurite added to 250 c.c. of bromthymol blue lactose agar just before pouring).

In studying the feces of 124 chronic invalids, plasma-coagulating staphylococci were found in 12 (9.7 per cent) in the following amounts: "moderate number," "moderate number," 1, 1, 2, 2, 2, 7, 7, 10, 10 and 14 millions per 100 grams of dry feces. There was an overgrowth of other organisms in 8 specimens (6.5 per cent).

There was a relationship to the presence of staphylococci in the upper respiratory tract, plasma-coagulating staphylococci being found in the pharynx in 11, and in both pharynx and nares in 9 of the 12 that showed them in the feces. This indicates that the organisms had been swallowed. Not all swallowed staphylococci, however, reach the feces because, of the 44 patients that showed plasma-coagulating staphylococci in the pharynx, only 11 showed them in the feces.

The method does not entirely eliminate other organisms but the number of other organisms is so reduced and the appearance of colonies of plasma-coagulating staphylococci is so characteristic that there is little difficulty in recognizing them. Enterococci are the most comon interfering organisms but the colonies are quite different from those of staphylococci. Five of the cultures of staphylococci isolated failed to clot plasma and are not included in the above calculations.

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From the Clinical Research Laboratory.
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ALLERGY FROM TIMBÓ (LONCHOCARPUS H.B.K.)

Report of a Case

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CONSIDERING the wide use of "timbó" (Lonchocarpus H.B.K.) and "cubé" (Derris Lour.) as insecticides, the paucity of reports of allergic manifestations in connection with these substances is rather surprising. Weston[1] has reported two cases of asthma due to Derris-root in flea powder.

We had the opportunity of studying a patient with allergy due to timbó who manifested both asthma and dermatitis of the contact type. The interest of this case lies in the demonstration of cross reactions between the atopens of Lonchocarpus (timbó) and Derris (cubé), plants of the Leguminosae family.

CASE REPORT

M. J. S., a Brazilian white male, aged 33 years, has been working as an engineer for three years, on the road-building service in the State of Amazonas (North Brazil). Until he started to work there, he had never suffered from asthma or eczema. He had had urticaria when he ate shrimps. Typhoid fever and pneumonia were the only illnesses he had had in the past. His mother had suffered from allergic rhinitis; two maternal aunts had asthma; one sister and two sons had chronic urticaria.

In the beginning of 1940 his work brought him into close contact with the flora of Amazon. His abode was located at a distance of some kilometers from where he worked and he was compelled daily to walk through dense shrubs and forests. The great number of mosquitoes of this region forced him to spray his house with a mixture of kerosene and timbó powder. He noticed first that he could not breathe well when he stayed in the rooms which had been sprayed. Without paying special attention to the fact he continued to use timbó, the only insecticide at his disposal.

In May, 1940, there appeared the first trouble which forced him to stop using the spray. At that time, inhalation of the above mixture would cause him to sneeze, followed by coryza, pruritus of the nose and conjunctiva, oppression on the chest, shortness of breath and wheezing. On stopping use of the insect powder, the patient was relieved of all symptoms. Although he no longer sprayed his rooms, he could not altogether avoid the effects of the mixture, for his colleagues, at whose houses he called, kept on spraying. Frequently he felt himself suddenly compelled to leave the houses of friends because of violent attacks of asthma.

At that time he experienced pruritus and reddening of the skin of his hands while working in the field. After a few days, there appeared small vesicles, followed by pruritus and eczematization of his hands and forearms. Although he suspected the toxic action of some plant, he was unable to identify it. His eczema healed after ten days' rest. On returning to work the eruptions reappeared, of the same character, but now spreading beyond the elbows. The patient came to Rio de Janeiro where we examined him. His dermatosis had healed during the journey.

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Physical Examination.—The examination did not reveal anything of interest. Kahn test was negative. He had a blood eosinophilia of 7 per cent. Urine and stool examinations were negative.

Skin Tests.—Scratch and intradermal tests were negative for foods, common inhalants, fungi and pollens. The scratch tests with a 1 per cent extract of Lonchocarpus nicou (timbó) root-powders and with a similar extract from Derris elliptica (cubé) were both positive 3 plus. The same tests were negative on the skin of three normal persons. The passive transfer tests were positive 2 plus on the skins of 2 non-atopic recipients. Scratch and intradermal tests with an extract made from Tephrosia, a genus of the Leguminous family and possessing also the insect-killing capacity, were negative. Also negative were the scratch and intradermal tests done with a standardized extract from pure rotenone. Patch tests, not covered, with crude extracts obtained from the leaves of Lonchocarpus and Derris, gave positive reactions of the same intensity for the two genera. After 24 hours there were intense erythema, distinct infiltration and vesiculation, within an area 3 centimeters in diameter. On the third day after performance of the patch test, an erythematous "flare-up" appeared on the backs of the two hands, which disappeared without vesiculation after 72 hours. The same tests were negative on the skins of 3 normal persons. Attempts to transfer the sensitivity in relation to the resinous principles of the two plants have not been successful. The patch test performed with Tephrosia, pure rotenone, Lithrea molleoides, and kerosene were negative. The contact tests with powdered leaves or roots of Lonchocarpus and Derris exhaustively treated with ether (tests performed with a 50 per cent ointment by weight of powder and petrolatum) were wholly negative after 24, 48, 72 hours and after 7 days.

DALE EXPERIMENTS

In order to be certain of the immunological relationship between the water soluble antigens of the two genera, Lonchocarpus and Derris, a series of experiments with the Dale test were conducted. Five guinea pigs were sensitized by means of a single intraperitoneal injection of a purified alum-precipitated extract. It was possible to inactivate completely a *Lonchocarpus nicou* sensitized uterine strip by means of an extract of *Derris elliptica* and vice-versa, in several cross neutralization experiments. Similar experiments were carried out with the species* *Lonchocarpus urucú Kill, L. maculatus DC., L. densiflorus Benth., L. floribundus Benth., L. sericeus H.B.K., L. denudatus Benth., L. spruceanus Benth., L. negrensis Benth.; Derris guiaanensis Benth., D. negrensis Benth., D. longifolia Benth.* All of them showed the presence of a common antigen.

Extracts from three species of the genus *Tephrosia Pers.* (*T. toxicaria Pers., T. brevips Benth., T. nitens Benth.*) and from pure rotenone, failed to produce contraction on the uteri sensitized to Lonchocarpus or Derris.

Precipitin tests were performed with the extracts used for sensitization in sera from the five guinea pigs, but the results were negative.

DISCUSSION

The simultaneous appearance, in our patient, of asthma and dermatitis of the contact type, must be considered as a mere coincidence. The eczematous eruptions have been found only in connection with the resinous substance of the Lonchocarpus and Derris leaves. The patch tests with leaf and root powder, exhaustively extracted with ether, did not produce any reaction. We must conclude that the eczematous eruptions cannot be ascribed to the atopen prin-

*All the species of the genus Lonchocarpus are designated here as "timbó" and are generally considered as containing rotenone.

ciple of the plants just considered. Besides, the aspect of the lesions is quite different from that described by Walzer and Albert.[2]

The existence of cross reactions between the atopenic principles of the two genera, Lonchocarpus and Derris, has been demonstrated by means of the Dale test. Other genera of the Leguminosae family have been shown incapable of neutralizing a Lonchocarpus or Derris sensitized uterine strip. The fact that our patient does not react to pure rotenone, one of the principal substances endowed with toxic action for cold-blooded animals, shows that the atopenic and contact principles of the two genera belong to different classes of substances. The other substances which are contained in timbó powder (deguelina, tephrosina, toxicarol) could not be investigated owing to the difficulty of obtaining them in the pure state. We have not been able to study the immunologic relationships of the resinous substances of the genera Lonchocarpus and Derris. The attempts artificially to sensitize the skin of three normal persons with its resin did not give conclusive results.

SUMMARY

The writer cites the case of a patient who became allergic, at the same time, to the atopenic and resinous principles of timbó, a plant belonging to the genus Lonchocarpus. The patient reacted specifically also to the two principles of the genus Derris (cubé). On studying the antigenic behavior of the atopenic principle of the genera Lonchocarpus and Derris by means of the Dale test, the writer was able to show the existence of cross reaction between the two plants. Pure rotenone was inactive in the cutaneous tests and indiferent to the uterine muscle of guinea pig sensitized to any of the above genera referred to.

We wish to express our thanks to Professor Mello Barreto and to Dr. Luiz de Mello Filho for the facilities they afforded us in the execution of this work.

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OBSERVATIONS ON THE HEMATOLOGIC ACTIONS OF ACETANILID AND ACETOPHENETIDIN IN THE DOG

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WITH THE TECHNICAL ASSISTANCE OF DOROTHY BLAIR

ACETANILID and acetophenetidin are widely employed as analgesic and antipyretic drugs. There is considerable interest in the possible hematologic side effects their administration may produce. It is apparent from the clinical and experimental literature that chronic ingestion of large doses of these drugs may cause cyanosis and anemia. The cyanosis has been attributed to methemoglobin or sulfhemoglobin, or in some cases to "p-aminophenol" pigments. The anemia appears to be of the hemolytic type. In the etiology of agranulocytosis acetanilid has been suspected in two cases and acetophenetidin in three cases. (For extensive bibliography and further discussion see references 1-5.)

The chief problems to be considered are the nature of the hematologic effects, the mechanisms of their production, and their relationship to dosage. More data are needed to clarify these problems, especially in regard to the correlation of peripheral blood and bone marrow changes. The choice of a suitable experimental animal for further studies requires consideration of possible differences in the response of various species. Certain quantitative differences are known to exist, as, for example, in susceptibility to formation of methemoglobin by acetanilid and acetophenetidin: the cat is most sensitive, man is approximately half as sensitive as the cat, the dog is half as sensitive as man, the rat is one-sixth as sensitive as the dog, and the rabbit and monkey are relatively insensitive.[6] Preliminary observations on the hematologic effects of acetanilid in the dog also suggested that this species was more susceptible to the development of anemia than the rat, rabbit, and monkey, but less sensitive than the cat. Therefore, for the present hematologic investigations, the dog was chosen as the most suitable animal, since it is moderately susceptible to the development of anemia and to methemoglobin formation. Dogs, furthermore, can be easily trained to lie quietly and to take the drugs in capsules without requiring a stomach tube, are sufficiently large to allow repeated blood examinations, and can be maintained satisfactorily for long periods under laboratory conditions. There are only a few studies of the hematologic effects of acetanilid and acetophenetidin on the dog; the best known are the experiments with acetanilid reported by Payne.[7]

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In the present investigation the effects of a wide range of dosages of acetanilid and acetophenetidin on peripheral blood have been observed, and an attempt has been made to correlate these with the bone marrow changes. The objective was to characterize the actions of toxic doses of the drugs and to establish as nearly as possible the minimal doses which produce significant effects.

METHODS

Adult healthy dogs were selected and arranged in groups containing approximately equal numbers of males and females. The diet consisted of a stock kennel ration of dog blocks in sufficient quantity to maintain or permit a gain in weight of control animals, and water was allowed ad libitum. All animals were trained to be tied down, and blood samples were taken only after the animals had been quiet for some time. Control values for each animal were established during several weeks prior to the administration of the drugs. The acetanilid and acetophenetidin (U.S.P.) were administered orally as a dry powder in capsules, once daily, late in the morning. Blood samples were obtained from the femoral artery early in the morning approximately twenty hours after administration of the drugs and sixteen to eighteen hours after feeding. An ammonium-potassium oxalate mixture[8] was used as anticoagulant. All hematologic examinations were started immediately after the blood samples were obtained.

TABLE I

EFFECT OF DAILY ADMINISTRATION OF ACETANILID IN THE DOG

DOG NO.	DAYS	DAILY DOSAGE MG./KG.	THb GM.%	ERYTHROCYTES IN MILLIONS	CELL VOLUME %	RETICULOCYTES %	LEUCOCYTES IN THOUSANDS
	0	0	13.9	5.31	41.4	1.7	13.55
9	52	9	16.5	6.12	50.0	0	10.50
	110	9	14.1	5.99	45.0		13.40
	0	0	16.3	5.85	50.1	0.5	10.89
10	52	9	14.5	5.48	48.0	0	11.53
	110	9	18.0	6.73	56.0		11.08
	0	0	14.2	5.85	46.8	0.2	8.62
	52	9	14.5	6.10	46.0	0	9.95
	80	9	14.4	6.86	45.0		9.90
15	42	18	15.1	6.96	47.0		11.35
	63	36	13.9	6.00	47.0	0	7.32
	77	72	11.6	5.48	37.0	0	12.20
	45	144	9.5	4.35	34.0	5.6	12.60
	0	0	15.1	5.89	45.1	1.5	11.14
	52	9	14.4	6.44	41.0		9.90
	80	9	14.1	6.44	45.0	0.1	9.58
19	42	18	16.2	6.45	51.0		10.42
	63	36	14.7	6.40	54.0	0.4	8.50
	77	72	15.4	6.60	52.0	0.4	9.12
	45	144	9.5	4.82	37.0	7.2	9.53

The methods used in this study to examine peripheral blood and bone marrow were described in a previous report on the normal hematology of the dog.[9] The mean values reported (Table I-*A*[9]) were employed to calculate the volume and color indices. Reticulocyte counts reported as zero indicate only that none were observed in the 500 erythrocytes counted, although some were

always present on the slide. The fragility of red cells to hypotonic saline was tested in the conventional manner.[10] Total hemoglobin, methemoglobin, and sulfhemoglobin were determined by the method of Evelyn and Malloy.[11] Plasma bilirubin was measured by the method of Malloy and Evelyn.[12] At the end of the experiments most of the animals were sacrificed, usually by decapitation, and tissues were obtained immediately for bone marrow examination.

The significance of the observed hematologic changes in peripheral blood was established largely by comparison of the control observations on each animal or group of animals with the subsequent experimental values. In parallel experiments in which four animals or more were employed in a group, all control determinations were pooled, and the mean with its standard error was calculated. The experimental values of the group at any given time interval during administration of the drugs were similarly treated. Statistical significance of the difference between the means was tested by Fisher's "t" test.[13] (This treatment was applied especially to the "Series 2" groups, Figs. 1 and 3.) The normal values reported for the eighty-one animals examined in this laboratory[9] and a smaller group of ten animals, followed for several months were also employed in a general way in interpreting the data. The myeloid-erythroid ratios calculated from the bone marrow counts (Table II) were also treated statistically as described above except that the control values in this case were derived from a separate group of animals.[9]

RESULTS

I. Acetanilid.—

Series 1.—Two dogs received acetanilid 9 mg. per kilogram daily for 147 days; the drug was discontinued for two weeks, and the animals sacrificed. Two other dogs received progressively increasing doses of acetanilid: 9 mg. per kilogram daily for 96 days, 18 mg. per kilogram for 51 days, 36 mg. per kilogram for 63 days, 72 mg. per kilogram for 77 days, and 144 mg. per kilogram for 45 days; i.e., 332 days of continuous medication. The hematologic data are summarized in Table I. The chronic administration of 9, 18, and 36 mg. per kilogram of acetanilid produced no significant changes in the peripheral blood. An increase in dosage to 72 mg. per kilogram resulted in a slight decrease in total hemoglobin, erythrocytes, and cell volume in Dog 15 but no change in Dog 19. A further increase in dosage to 144 mg. per kilogram, however, produced in both dogs a moderate decrease in total hemoglobin, erythrocytes, and cell volume, and a significant reticulocytosis. In this entire series no significant changes occurred in the total or differential leucocyte counts. There was no change in fragility to hypotonic saline and no cumulation of methemoglobin or sulfhemoglobin. The color and volume indices remained normal. The animals maintained or gained weight.

Series 2.—Acetanilid 36 and 72 mg. per kilogram. Two groups of four animals each were on experiment for 78 days; one group received 36 mg. per kilogram per day and the other group 72 mg. per kilogram per day. Blood examinations were made each week. The data on peripheral blood are summarized in Fig. 1. No consistent change occurred in total hemoglobin, erythrocytes, or cell volume in the 36 mg. per kilogram group, although a few of the values for erythrocytes were significantly reduced. The color and volume indices

remained normal. The values for total hemoglobin, erythrocytes and cell volume for the 72 mg. per kilogram group showed phasic decreases which in general were quite significant. At the end of the experiment the mean values were significantly reduced. The color index remained normal but the volume index increased slightly. In both groups there was a small increase in the reticulocyte

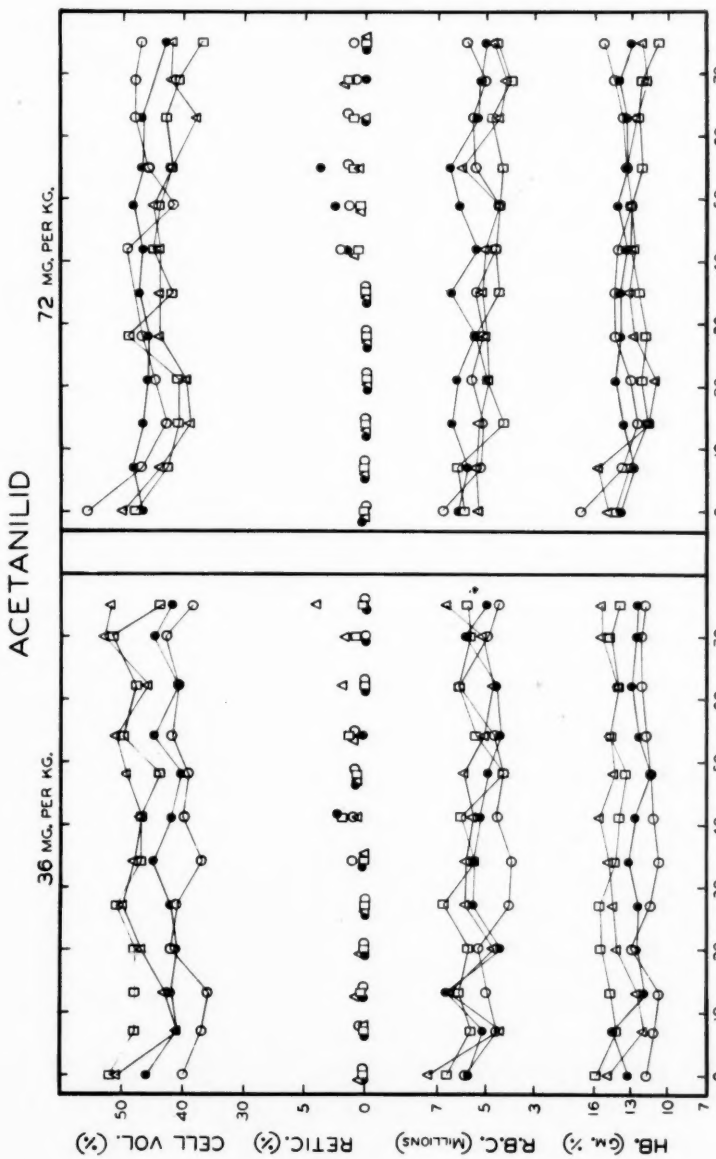

Fig. 1.—Effect of daily administration of acetanilid 36 mg. per kilogram (left) and 72 mg. per kilogram (right) on peripheral blood. Each character represents an individual dog.

count beginning about the fortieth day. No significant deviation was observed in the total or differential leucocyte count, and no cumulation of methemoglobin or sulfhemoglobin occurred. All animals either maintained or gained weight.

Series 3.—Acetanilid in large doses. Experiments were carried out on twelve dogs using, in most instances, a dose of 250 mg. per kilogram per day

for five to nine days. Occasionally the experiment was initiated by two 250 mg. per kilogram doses on the first day. The peripheral blood was usually examined daily and administration of the drug stopped after a severe anemia developed. Some animals were sacrificed at this time for bone marrow examination; others were followed to complete recovery of the peripheral blood picture. In several instances the latter type of experiment was repeated one or more times on the same animal.

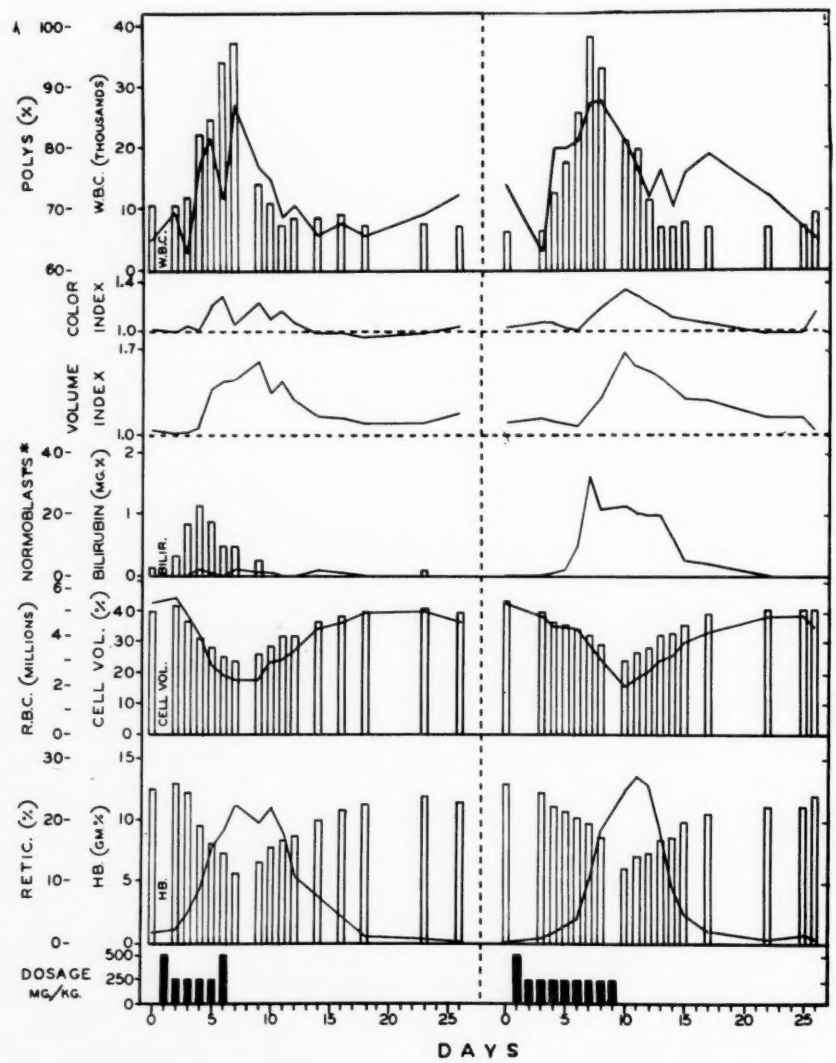

Fig. 2.—Effect of the administration of large doses of acetanilid on peripheral blood: the first experiment (left), and third experiment (right) on the same dog. The inner ordinate refers to the bars. The number of normoblasts are those observed in counting 200 leucocytes; the leucocyte counts are uncorrected and are thus too high by the number of normoblasts present.

Two examples of the effects produced with these large doses of acetanilid on one dog are shown in Fig. 2. In this animal the experiment was repeated four times, the first and third experiments being shown. In the first experi-

ment this dog received 500 mg. per kilogram on the first day, 250 mg. per kilogram on the second, third, fourth, and fifth days, and 500 mg. per kilogram on the sixth day. It is evident that by the fourth day a definite decrease in total hemoglobin, erythrocytes, and cell volume had occurred which reached a minimum on the seventh day. The reticulocyte response began on the third day. When administration of the drug was stopped recovery was rapid and normal values were approximated by the twenty-third day. The anemia was macrocytic and tended to be slightly hyperchromic. The development of the anemia was associated with a mild bilirubinemia, and some increase in the number of normoblasts in the peripheral blood.* During the last few days of administration of the drug and associated with the development of the anemia, there occurred a marked leucocytosis and an increase in the percentage of polymorphonuclear neutrophils with a definite shift toward more immature forms as indicated by a decrease in the polynuclear count. Younger forms of lymphocytes also appeared. The leucocytosis subsided early in the recovery period. The third experiment, Fig. 2, and the fourth experiment were in general a reproduction of the events of the first. The drug was administered for nine and five days, respectively. The chief variation from the first experiment was the appearance of a greater number of normoblasts in the blood. The second experiment on this animal differed only quantitatively in that there was a lesser tendency to the development of anemia.

The results shown in Fig. 2 may be taken as representative of this series. Some variation was noted in the number of daily 250 mg. per kilogram doses required to produce a severe anemia, although five to ten days were usually sufficient. Similar experiments were also made on four splenectomized dogs. The hematologic effects were essentially the same and no definite difference in susceptibility or rate of recovery was noted. No cumulation of methemoglobin or sulfhemoglobin was observed in any of these experiments.

Effect of Acetanilid on Bone Marrow.—Examination of femur sections and imprints of the marrow from dogs in Series 1 (9 mg. per kilogram) revealed normal cellularity with a slightly increased percentage in the erythroid series for Dog 9 (confined chiefly to the polychromatic normoblasts), and no change in Dog 10. The femur marrow imprints of Dog 15 (progressive 9 to 144 mg. per kilogram) showed an increased erythroid percentage with a myeloid-erythroid ratio of 0.904. Femur sections on both Dogs 15 and 19 showed an increase in erythroid cells and some hyperplasia.

The result of differential rib marrow counts for Series 2 are shown in Table II. With the 36 mg. per kilogram dose the average myeloid-erythroid ratio for the rib was 0.946 and for the femur 0.827, representing a significant decrease from the control series. This was associated with an increase in the pronormoblasts and orthochromatic normoblasts. The femur marrow sections also indicated an increase in the erythroid series; hyperplasia was evident in two animals, no change in one, and an apparent hypoplasia in one. The effect of the 72 mg. per kilogram dose was even more significant. The average myeloid-erythroid ratio was reduced to 0.817 for the rib preparation and 0.647 for the femur. The increase in erythroid percentage occurred chiefly in the polychromatic and orthochromatic normoblasts. The histologic sections also

*We have frequently noted occasional normoblasts in the peripheral blood of control dogs.

TABLE II

EFFECT OF ACETANILID AND ACETOPHENETIDIN ON DIFFERENTIAL BONE MARROW COUNTS FROM RIB SMEARS IN THE DOG

TYPE OF CELL	CONTROLS* 8 DOGS	ACETANILID 36 MG./KG. 4 DOGS	ACETANILID 72 MG./KG. 4 DOGS	ACETO-PHENETIDIN 60 MG./KG. 4 DOGS	ACETO-PHENETIDIN 120 MG./KG. 5 DOGS
Myeloblasts	0.2-1.0 0.6	0.4-0.7 0.6	0.5-1.0 0.8	0.7-1.5 1.1	0.4-1.9† 1.0‡
Promyelocytes	0.7-2.8 1.6	0.3-1.4 1.0	0.5-0.9 0.7	1.0-1.5 1.2	0.7-2.3 1.5
Myelocytes	2.7-10.0 6.0	1.9-3.5 2.8	2.0-4.1 2.6	3.3-5.7 4.2	3.6-8.1 5.8
Metamyelocytes	1.1-4.6 3.4	1.1-4.3 2.7	2.0-3.6 3.0	2.8-5.2 3.4	1.6-4.7 3.6
Bands, Neutrophilic	6.8-17.4 11.7	9.3-10.2 9.8	6.7-9.8 8.0	7.6-12.9 9.8	6.4-11.2 8.3
Segmenters, Neutrophilic	16.8-44.2 30.1	22.3-29.6 26.8	21.4-29.3 25.9	19.8-27.9 22.6	21.4-33.1 24.5
Eosinophils	0.4-3.8 2.0	1.8-4.8 2.7	0.9-1.9 1.4	0.7-2.2 1.5	0.9-2.8 1.7
Monocytes	0.0-0.3 0.2	0.0-1.0 0.1	0.0-0.1 0.0	0.0-0.3 0.1	0.0-0.3 0.1
Lymphocytes	0.2-2.7 0.9	0.7-2.0 1.2	0.9-2.3 1.5	0.5-0.7 0.6	0.5-1.5 0.9
Pronormoblasts	0.2-1.2 0.6	1.3-1.4 1.4	0.9-1.7 1.4	1.0-1.4 1.2	0.7-1.6 1.1
Normoblasts, Basophilic	6.4-9.9 7.8	7.1-8.2 7.5	5.6-9.8 7.4	8.5-9.3 8.9	9.9-13.9 11.6
Normoblasts, Polychromatic	11.0-26.0 16.4	17.2-19.2 18.1	20.3-22.4 21.3	18.7-21.7 19.8	14.3-23.9 20.8
Normoblasts, Orthochromatic	8.9-25.8 17.4	18.9-27.8 23.9	17.3-28.5 24.5	18.1-27.6 24.2	10.8-24.8 17.7
Megakaryocytes	0.0-1.4 0.5	0.2-0.3 0.2	0.0-0.4 0.2	0.0-0.2 0.1	0.0-0.3 0.2
Unclassified	0.8-1.4	1.0-1.9	1.1-1.8	1.1-1.5	0.8-1.5
M-E Ratio Rib	0.6-1.78 1.37	0.83-1.16 0.946	0.68-1.08 0.817	0.75-1.09 0.836	0.77-1.17 0.935
M-E Ratio Femur Imprints	1.06-2.39 1.610	0.70-1.05 0.827	0.50-0.73 0.647	0.68-0.96 0.768	0.67-1.48 1.029

*See reference 9.
†Range.
‡Mean.

revealed an increase in the erythroid series. Hyperplasia of the marrow was evident in three animals with no change in the fourth.

A number of bone marrow observations were made on animals from Series 3, the animals which had received large doses of acetanilid sufficient to produce a peripheral blood picture similar to that demonstrated in Fig. 2. For example, a dog that had received 250 mg. per kilogram of drug per day for six days was sacrificed on the eleventh day with peripheral blood values as follows: total hemoglobin 7.5 Gm. per cent, erythrocytes 3.13 million, cell volume 30 per cent, reticulocytes 33.3 per cent, color index 1.01, and volume index 1.31. The bone marrow differential counts showed a marked decrease of the myeloid-erythroid ratio with values of 0.451 for rib and 0.394 for femur. An increase in all of the erythroid series was evident, but was especially marked in the polychromatic normoblasts (40.6 per cent). Both the white and red series of cells were complete. Histologic sections showed hyperplasia of the femur marrow.

II. Acetophenetidin.—

Series 1.—Acetophenetidin was administered to six dogs in 15 mg. per kilogram doses for 43 to 150 days. Five of these animals were then continued on 30 mg. per kilogram for 16 to 102 days; then four of these animals continued on 60 mg. per kilogram for 39 to 46 days; and finally two of the animals were continued on 120 mg. per kilogram for 28 days.

Table III

Effect of Daily Administration of Acetophenetidin in the Dog

DOG NO.	DAYS	DAILY DOSAGE MG./KG.	THb GM. %	ERYTHROCYTES IN MILLIONS	CELL VOLUME %	RETICULOCYTES %	LEUCOCYTES IN THOUSANDS
27	0	0	15.6	6.58	48.5	0	18.30
	76	15	18.7	7.11	44.0	0	8.25
26	0	0	13.7	6.01	41.5	0.2	9.74
	76	15	11.9	5.19	36.0	0	11.40
	71	30	11.5	5.01	35.0	0.4	20.83
28	0	0	15.4	7.23	47.5	0	12.60
	133	15	13.9	6.72	56.0	0.6	13.05
	51	30	14.0	6.47	50.5	1.1	11.70
	45	60	14.2	7.13	49.0	0.1	10.33
29	0	0	14.4	6.28	46.0	0.01	17.17
	96	15	12.5	4.95	37.0	1.0	14.15
	97	30	12.1	5.02	40.0	0.7	17.83
	45	60	10.5	4.70	35.0		12.78
P-1	0	0	15.5	6.38	46.0	0.3	10.30
	41	15	16.2	7.75	48.0	0.6	12.55
	14	30	15.5		47.0	0.4	8.23
	35	60	15.6	5.95	44.0	2.1	9.25
	27	120	14.2	5.14	42.0	0	7.40
P-2	0	0	15.8	5.95	41.0	0.6	10.19
	41	15	16.6	8.12	50.0	0.9	10.70
	14	30	13.9		44.0	0.2	12.60
	35	60	17.3	6.29	51.0	0.6	9.18
	27	120	17.1 ?	5.68	45.0	0.8	8.78

The data as summarized in Table III did not demonstrate any consistently significant change in total hemoglobin, erythrocytes, and cell volume, although certain of the individual values were below the control level. In this entire series no significant changes occurred in the total or differential leucocyte count. There was no change in fragility to hypotonic saline or cumulation of methemoglobin or sulfhemoglobin. The color and volume indices remained normal, and the animals maintained weight.

Additional data to that shown in Table III suggested that daily doses of 60 and 120 mg. per kilogram may produce a temporary decrease in total hemoglobin and erythrocytes. Further experiments to test this are described in Series 2.

Series 2.—Acetophenetidin 60 and 120 mg. per kilogram. Four dogs received 60 mg. per kilogram of the drug per day for 70 days, and five additional dogs received 120 mg. per kilogram per day for 70 days. The weekly hematologic observations are presented in Fig. 3. During the administration of the 60 mg. per kilogram dose there was a significant decrease in total hemoglobin, erythrocytes, and cell volume within the period of fifteen to forty days, but thereafter these values progressively increased and were not significantly below the control values at the end of the experiment. With the 120 mg. per kilogram

dose, a similar significant decrease in total hemoglobin, erythrocytes, and cell volume occurred during the period of fifteen to fifty days which was followed by some increase in the values. At the end of the experiment some of the values were below the control level, but the average values were not significantly different from the control values. In both groups some increase in reticulocytes

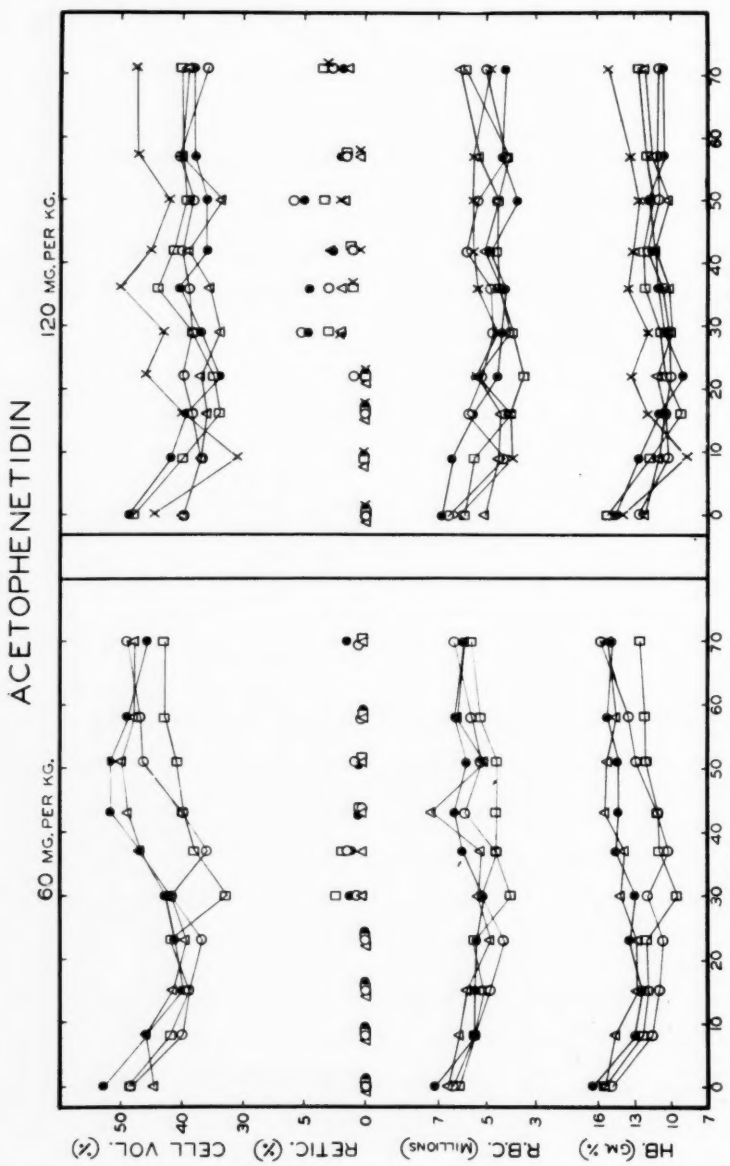

Fig. 3.—Effect of daily administration of acetophenetidin 60 mg. per kilogram (left) and 120 mg. per kilogram (right) on peripheral blood. Each character represents an individual dog.

occurred after twenty-one to twenty-eight days, which was significant in the 120 mg. per kilogram group. The volume and color indices remained within normal limits. No significant change was observed in the total or differential leucocyte counts, and there was no cumulation of methemoglobin or sulfhemoglobin. All animals either maintained or gained weight.

Series 3.—Acetophenetidin in large doses. Acetophenetidin was administered in daily doses of 250 mg. per kilogram for fifteen days to one dog, and for one hundred days to two dogs, and in doses of 200 mg. per kilogram for seventy-six days to two dogs. The two experiments shown in Fig. 4 on two different dogs receiving 250 mg. per kilogram daily for one hundred days are representative. Both experiments demonstrate some effect on the peripheral blood. The total hemoglobin, erythrocytes, and cell volume showed phasic decreases with a

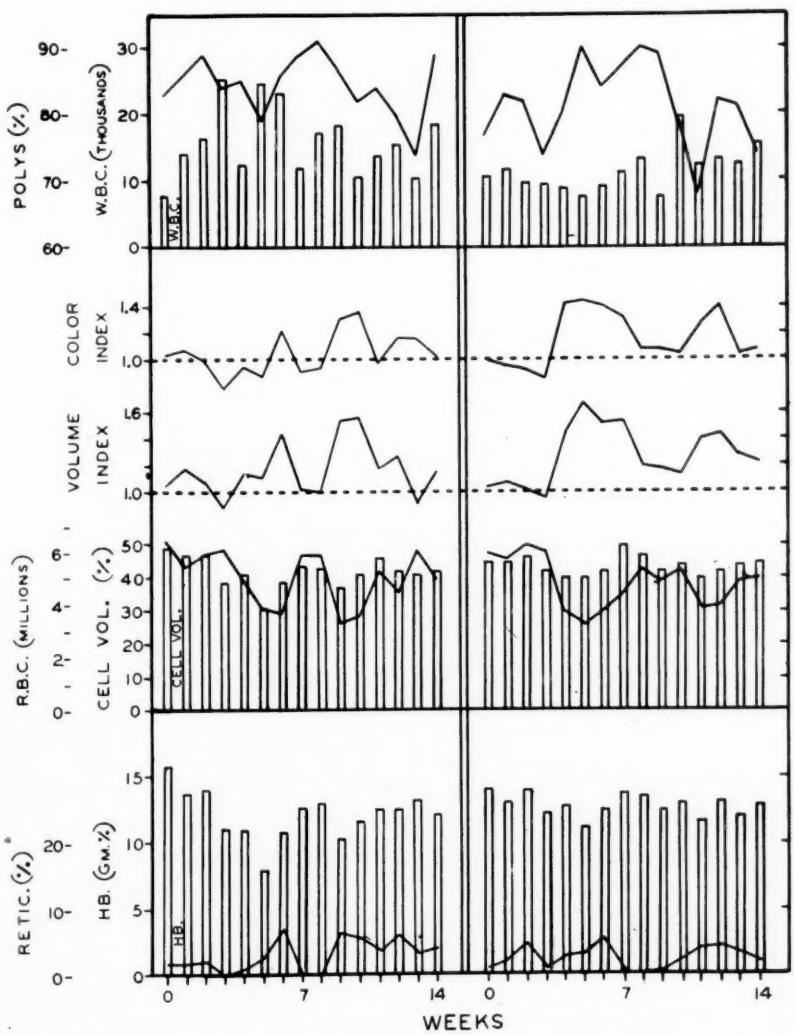

Fig. 4.—Effect of daily administration of acetophenetidin in doses of 250 mg. per kilogram on the peripheral blood of two dogs. The conventions are the same as in Fig. 2 except that time is in weeks.

subsequent trend toward normal values. A decrease in the erythrocyte counts was associated with reticulocytosis. During the phases of anemia there was an increased volume index and a small increase in color index. No consistent effect upon the total or differential leucocyte counts was observed. No cumulation of methemoglobin or sulfhemoglobin occurred. In general the animals maintained or gained weight.

Effect of Acetophenetidin on Bone Marrow.—In Series 1 the counts on femur imprints of the bone marrow from Dogs 26 (15 to 30 mg. per kilogram) and 29 (15, 30, and 60 mg. per kilogram) showed some increase in the polychromatic normoblasts with some decrease in the orthochromatic normoblasts resulting in no significant decrease of the myeloid-erythroid ratio. No change was observed in the femur sections. However, in Dogs P-1 and P-2 in which the dose was increased more rapidly (progressive 15 to 120 mg. per kilogram) the femur imprints showed a marked increase in the pronormoblasts and polychromatic normoblasts; the myeloid-erythroid ratio was significantly decreased to 0.386 and 0.549. Histologic sections of the femur also indicated an increase in percentage of erythroid cells; and the cellularity appeared normal.

The differential rib marrow counts for Series 2 are shown in Table II. With the 60 mg. per kilogram dose the myeloid-erythroid ratio was 0.836 for the rib, and 0.768 for the femur. Some increase in the percentage of pronormoblasts and orthochromatic normoblasts was evident. Femur sections were available on only three animals: two showed some hyperplasia, and the other normal cellularity. For the 120 mg. per kilogram group the average myeloid-erythroid ratios were 0.935 for the rib and 1.029 for the femur. Some increase was evident in the percentage of all the erythroid series except orthochromatic normoblasts. The cellularity of the femur marrow appeared normal in three dogs, and hyperplastic in one. Compared to the effects produced by 60 mg. per kilogram acetophenetidin, the differential counts on the 120 mg. per kilogram group showed a somewhat greater increase in the younger erythroid series, although the total erythroid percentage increase was less. In both cases the myeloid-erythroid ratios were significantly reduced.

Bone marrow studies made on the two dogs of Series 3 shown in Fig. 4 revealed the myeloid-erythroid ratios to be 0.704 and 0.600 for rib smears, and 0.514 and 0.602 for femur imprints. In both cases there was a general increase in the percentage of erythroid cells which was most marked in the polychromatic and orthochromatic normoblasts. Both the white and red series of cells were complete. Histologic sections also revealed some hyperplasia with evidence of increased erythroid activity.

III. Methemoglobin Formation.

It has been stated that with all of the repeated single daily dosages of the drugs employed in this study there was no cumulation of methemoglobin. This has meant that when the blood was examined twenty hours after administration of the drugs either no methemoglobin or only traces were present. A sufficient dose of either acetanilid or acetophenetidin produces a temporary methemoglobinemia in the dog. However, the amount of drug must exceed a certain threshold or minimal dose to produce significant amounts (i.e., 0.4 Gm. per 100 c.c. or twice the error of the method). According to Lester's data,[6] with which we are in essential agreement, the average threshold dose for acetanilid is about 20 mg. per kilogram, and for acetophenetidin it is a little more than 30 mg. per kilogram. The average maximal methemoglobin formation in dogs with normal total hemoglobin values for the various doses of drugs used in this report have been estimated for the lower range from the data of Lester, and for the higher range from our own data, and are as follows:

ACETANILID		ACETOPHENETIDIN	
DOSE MG./KG.	MHb GM./100 C.C.	DOSE MG./KG.	MHb GM./100 C.C.
9	0.1		
18	0.5	15	0.1
36	1.6	30	0.4
72	4.3	60	1.9
144	5.8	120	3.3
200	6.1	200	4.3

The duration of the methemoglobinemia is related to the size of the dose, but even with the largest doses employed in these studies no more than traces were present after twenty hours, and consequently no cumulation on daily doses was noted. The maximal amount of methemoglobin formed with the larger doses is dependent on the total hemoglobin concentration.[22] (A more complete description of our data on methemoglobin formation will appear in a subsequent publication.)

DISCUSSION

The data on acetanilid indicate that the daily administration of 9 or 18 mg. per kilogram doses of the drug produce no significant effect on peripheral blood. Similarly the effects on peripheral blood of 36 mg. per kilogram of the drug daily are probably not significant; although examination of the bone marrow indicates a significant increase in erythropoiesis. The effects upon peripheral blood of 72 mg. per kilogram of acetanilid daily are significant in indicating a tendency toward development of a mild anemia with some reticulocytosis. This observation gains further weight due to the fact that the bone marrow shows a more significant increase in erythroid activity. A moderate degree of anemia is produced by daily doses of 144 mg. per kilogram. The administration of large doses of acetanilid, 250 mg. per kilogram daily for five to ten days, clearly demonstrates the production of a severe hemolytic anemia. When the erythrocytes, total hemoglobin and cell volume decrease, there is a reticulocytosis, an elevation of plasma bilirubin, and a marked increase in erythroid activity in the bone marrow. The appearance of an increased number of normoblasts as well as less mature leucocytes in the peripheral blood also indicates a marked bone marrow response. The anemia is macrocytic with a tendency to be hyperchromic probably due largely to the reticulocytosis. The peripheral blood picture returns rapidly to normal when administration of the drug is stopped. Such experiments have been repeated several times on the same animal and indicate that no depression of marrow activity occurs. The minimal daily dose tested which produced significant effects on peripheral blood was 72 mg. per kilogram.

Acetophenetidin in daily doses of 15 and 30 mg. per kilogram appears to produce no consistent effect upon the peripheral blood. Daily doses of 60 mg. per kilogram cause a mild anemia which is temporary since the peripheral blood picture slowly returns toward normal even with continuation of the drug. At the same time the bone marrow shows a significant increase in erythroid activity. Daily doses of 120 mg. per kilogram produce a slightly greater and somewhat more persistent anemia and a significant reticulocyte response. Although the bone marrow shows evidence of increased erythropoiesis the change is not greater than that observed on the 60 mg. per kilogram dose. However, when the dosage is progressively and rapidly increased to 120 mg. per kilo-

gram there is a greater inversion of the myeloid-erythroid ratio. The continued administration of large doses of acetophenetidin, 250 mg. per kilogram daily for several weeks, does not produce an anemia comparable to that observed with acetanilid. Rather the effect is a phasic decrease in total hemoglobin, erythrocytes, and cell volume followed by recovery. However, the bone marrow shows considerable evidence of increased erythropoiesis. When anemia occurred, it was macrocytic and tended to be hyperchromic. As with acetanilid, the evidence indicates that the anemia is of hemolytic origin.

No significant changes in the total or differential leucocyte counts were observed with either drug except for the leucocytosis during the severe anemia produced by large doses of acetanilid. In the observations on bone marrow, no abnormal cells were seen, and all normal types of cells were present.

Therefore, it appears that in the dog daily doses of these drugs in excess of 36 mg. per kilogram of acetanilid and probably 60 mg. per kilogram of acetophenetidin are required to produce a sustained decrease in the erythrocytes in the peripheral blood. There is no doubt that sufficiently large or toxic doses can produce definite evidence of accelerated erythrocyte destruction as judged by the effects on peripheral blood and responses of the bone marrow. The actions of acetanilid and acetophenetidin are qualitatively similar; however, acetanilid is quantitatively more active in producing a hemolytic anemia. This difference may be due to differences in solubility and absorption, or in the manner in which the drugs are metabolized.

Quantitative comparison of our data on the activity of acetanilid and acetophenetidin in producing hemolytic anemia in the dog with the effects of this activity on man cannot yet be made because of the lack of controlled experiments and bone marrow observations on man. The best approximation, but based only on total hemoglobin, is from the data of Lester[14] which suggests that the dog may be somewhat less susceptible to the development of anemia than man. On the other hand the response of the dog to acetanilid is appreciably greater than that reported for the rat[15] and the monkey,[1] although the qualitative effects on peripheral blood in both species and on the marrow in the monkey appear similar. The hematological actions of acetanilid on peripheral blood in the two dogs reported by Payne[7] are similar to ours, with the bone marrow showing hyperplasia due to an increase in both erythroblastic and myeloid elements. These bone marrow findings on experimental animals resemble those in man in the only case report found. Lundsteen et al[4] reported observations on a patient who had taken large doses of both acetanilid and acetophenetidin (daily doses amounting to 1.5 Gm. of each drug); the marrow was hyperplastic with increased leucopoiesis, megakaryopoiesis, and markedly increased normoblastic erythropoiesis.

Both acetanilid and acetophenetidin in sufficient dosage are capable of producing methemoglobinemia in the dog. As is well known, however, methemoglobin is rapidly reconverted to hemoglobin, which explains why we found none 20 hours after administration of the drugs. It is worth noting that no cumulation occurred with repeated single daily doses. The dosage and frequency of dosage causing cumulation in man has been discussed by Lester.[14] We have not observed the formation of significant amounts of sulfhemoglobin with these drugs in the dog, nor any cumulation on chronic administration. Neither acetanilid nor acetophenetidin appear to form methemoglobin directly,

but only after conversion in the body to other substances. It has been suggested that p-aminophenol or phenylhydroxylamine may be among the intermediates. (For further discussion and references see 16 and 17.) In our experience p-aminophenol is capable of forming methemoglobin directly and rapidly, and is more active in producing anemia than acetanilid or acetophenetidin. This, of course, suggests that if p-aminophenol is formed, it can play a definite role in the observed hematological effects. It is quite possible that methemoglobinemia per se may also act as a stimulus to erythropoiesis, since we have shown that a reduced venous oxygen saturation occurs during methemoglobinemia.[18]

When sufficiently large doses of these drugs are administered to produce an anemia, our evidence indicates that it is of the peripheral hemolytic type as characterized by reticulocytosis, increased plasma bilirubin, and hyperplasia of the bone marrow with inversion of the myeloid-erythroid ratio. The peripheral blood picture rapidly returns to normal when the administration of the drugs is stopped. In acute experiments, splenectomy did not appear to influence the development of the anemia with acetanilid nor the speed of recovery. We have been able to reproduce an almost identical peripheral blood-and-bone marrow picture by chronic bleeding alone.

No definite conclusion has yet been reached concerning the exact mechanism of the hemolytic action of acetanilid and acetophenetidin. These drugs do not appear to be direct hemolysins in ordinary concentrations. However, preliminary experiments indicate that they can accelerate saponin lysis of washed red cells (method of Ponder[19]). It is not unlikely that the postulated metabolic breakdown products, p-aminophenol and phenylhydroxylamine, may also be concerned in the hemolytic action. The fact that acetanilid and acetophenetidin can produce methemoglobinemia could easily lead to the speculation that this action may be related to the process of hemolysis. Brownlee[20] has reported increased urinary porphyrin excretion in rats following administration of acetanilid and acetophenetidin. The increased porphyrin excretion was identified as due largely to coproporphyrin III. It has been suggested that this represents an abnormal pathway for the degradation of hemoglobin, possibly through methemoglobin.[20, 21] Rimington[21] has noted a correlation between the action of aromatic amino compounds to increase porphyrin excretion and to form methemoglobin. However, increased porphyrin excretion did not follow sodium nitrite administration. We have also noted some correlation between the activities of a series of aromatic amino compounds to produce methemoglobinemia and hemolytic anemia. The data of this report also indicate that the doses of acetanilid and acetophenetidin producing definite hemolytic effects exceed the threshold dose for methemoglobin formation. In spite of these inferences, however, and on the basis of work now in progress, it is our view that the ability of a drug to produce methemoglobinemia and hemolytic anemia may be only coincident actions dependent on certain physical and chemical properties. At least the production of methemoglobin per se cannot be accepted as a part of the hemolytic process without more direct proof.

SUMMARY

1. The hematological effects of a wide range of dosages of acetanilid and acetophenetidin on the peripheral blood and bone marrow of dogs are described.

2. The chronic administration of acetanilid up to 36 mg. per kilogram per day caused little or no abnormal hematological changes in dogs.

3. Large doses of acetanilid produce a hemolytic anemia with no evidence of depression of bone marrow. Recovery is rapid and apparently complete on cessation of administration of the drug.

4. The daily administration of acetophenetidin produces the same qualitative effects, but quantitatively the drug is less active than acetanilid.

5. Both drugs produce a temporary methemoglobinemia when certain threshold doses are exceeded. However, in the dosage range employed, no cumulation of methemoglobin was observed with repeated daily administration.

6. The possible mechanisms of the hematological actions are discussed.

The authors are grateful to Dr. J. L. Schwind of the Department of Anatomy for many helpful suggestions.

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THE EFFECT OF ESTRONE ON ANAEROBIC GLYCOLYSIS OF THE UTERUS OF THE RAT IN VITRO

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THE study of the estrogens has reached a stage at which their gross effects on organs need to be understood in terms of a fundamental change brought about by these substances in the biochemistry of a single cell. Such an understanding of the basic mechanism by which estrogens act not only would be of theoretic interest but would facilitate greatly the rational therapeutic use of these substances. A number of studies have been made with this in mind, among these a series of investigations on the effect of estrogens on the respiration of various tissues, particularly uterine tissue. In the following report, I shall deal only with anaerobic glycolysis, since it has been found that this process usually showed a greater and more rapid change than did the aerobic respiration in animals treated with estrogens.

Kerly[1] reported that the rate at which the uterus of the rat in vitro produced lactic acid was doubled during proestrus, as compared with that at diestrus or in uteri from spayed animals. She[2] showed that the rate of anaerobic glycolysis of uteri from spayed rats into which 2.5 micrograms of estrone in 1 per cent alcohol had been injected twenty-four to thirty-six hours previously was double that of uteri from rats into which estrone had not been injected. When estrone was added to the uterine tissue in vitro, however, no effect of 0.1, 2 or 25 micrograms of estrone was observed. However, her observations on the effect of estrogens in vitro were complicated by the side effects of the solvent.

Carroll[3, 4] reported a study of the effects that injection of large amounts (50 micrograms) of estradiol-2-propionate produced on anaerobic glycolysis of uteri of spayed rats. He also found that uteri taken from animals into which this estrogen had been injected twenty-four hours before produced lactic acid anaerobically at a higher rate than did uteri from spayed controls into which estradiol-2-propionate had not been injected, although the difference was not as great as that reported by Kerly. Endometrium and muscle of the uterus were studied separately and stimulation occurred in both.

The purpose of this study was to investigate further the effect of estrone added to uterine tissue in vitro under anaerobic conditions and to determine if possible whether the increased rate of anaerobic glycolysis of uteri from rats treated with estrogens is a primary effect of estrogens on the cells of the uterus or merely a result of their otherwise stimulated condition.

METHOD

Rats.—The uterine tissue used in these experiments was taken from white rats spayed at the age of three months. The sensitivity of the rats to estrone

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was standardized in the following way: following spaying, rats were rested one week, then smeared one week to determine whether spaying was complete. If these rats showed absence of estrous cycles, 1 microgram of estrone in 1 c.c. of olive oil then was injected, according to the usual assay procedure. Only rats positive for this dose were used. Following a week's rest 0.5 microgram of estrone was injected into these rats and only those negative for this dose were retained. The rats were used for experiments during the following week, the fifth after spaying.

Preparation of tissue.—Rats were killed by a blow on the head. The uterus was dissected out immediately, slit lengthwise and placed in warm phosphate buffer of pH 7.4. One horn of each uterus was used as a control while the substance to be tested was added to the other horn.

Bicarbonate Ringer's solution of the following composition:

96 parts	NaCl	0.15 M.
2 parts	KCl	0.15 M.
2 parts	$CaCl_2$	0.15 M.
20 parts	$NaHCO_3$	0.15 M. (Just acid to phenolphthalein)
2.4 parts	Glucose	10 per cent

was measured into the experimental flasks from a storage vessel containing an atmosphere of 95 per cent of nitrogen and 5 per cent of carbon dioxide.

Dilutions of estrone* were made up in bicarbonate Ringer's solution, 0.1 microgram in each cubic centimeter or less as a solution, higher concentrations as a fine suspension.† All solutions were made up with double distilled water, the second distillation having been carried out in a glass still. The pH of all solutions was checked at the beginning and end of each experiment with a Beckman glass electrode. The pH of the solutions used was 7.1 to 7.3. The activity of the solutions of estrone was checked by bio-assay.

Tissues were weighed quickly on a Roller-Smith torsion balance and placed in the experimental flasks. The flasks were attached to their manometers and placed in the thermostatically controlled water bath at 38 ± 0.1° C. A gas mixture of 95 per cent of nitrogen and 5 per cent of carbon dioxide, freed from oxygen by being passed over hot reduced copper wire and saturated with water vapor, was passed through the air space in the experimental vessels for ten minutes. Vessels then were closed and readings made in the usual way[5] every half hour. The $Q\,{}^{N_2}_{G}$ (the rate of anaerobic production of lactic acid expressed as cubic millimeters of carbon dioxide formed per hour per milligram dry weight of tissue) was calculated. Values for $Q\,{}^{N_2}_{G}$ in the tables are averages of these rates for each half hour.

Direct determinations of the total lactic acid produced during the experiment were made by the method of Barker and Summerson.[6]‡

*Crystalline estrone was donated by Parke, Davis and Company.

†I wish to express my gratitude to Dr. H. L. Mason, who made up all estrone solutions and suspensions.

‡I wish to express my indebtedness to Dr. J. L. Bollman and Dr. E. V. Flock, under whom this work was carried out, for their valued suggestions. Technical assistance was kindly given by Evelyn Cunningham, Henrietta Schasch, Cecily Barker and Emery Van Hook.

RESULTS

The average rate of anaerobic glycolysis of untreated uteri from spayed rats was 7.0 ± 1.4, a value comparable to that obtained by other workers for uteri of spayed rats (Kerly,[1] 11; Kerly,[2] 7; Carroll,[3, 4] for endometrium only, 6.1). The rate of production of lactic acid did not show any tendency to fall off during the four to six hours of the experiments. Production of lactic acid determined by the direct method of Barker and Summerson[6] at the end of the experiments averaged 23.9 ± 2.3 micrograms per milligram of tissue per hour, which is in reasonably close agreement with the lactic acid calculated from the total carbon dioxide evolved from bicarbonate, 23.4 ± 4.7 micrograms per milligram of tissue per hour. There was no evidence in any experiment to suggest that any substance other than lactic acid was liberating carbon dioxide during anaerobic glycolysis.

When 5 micrograms of estrone in 1 c.c. of bicarbonate Ringer's solution were injected into rats twenty-four hours before the removal of the uterus,

TABLE I

THE RATE OF ANAEROBIC GLYCOLYSIS ($Q_G^{N_2}$) OF UTERINE TISSUE OF SPAYED RATS IN DIFFERENT CONCENTRATIONS OF ESTRONE, IN VITRO

UNINJECTED RATS					INJECTED RATS	
WITHOUT ESTRONE	WITH ESTRONE, MICROGRAMS PER CUBIC CENTIMETER				WITHOUT ESTRONE	WITH ESTRONE, 0.01 MICROGRAM PER CUBIC CENTIMETER
	5	0.1	0.01	0.001		
5.8					6.3	
5.3					8.4	
6.6					10.5	9.0
6.6					8.6	8.7
6.6					10.8	10.9
8.3					11.6	
8.3					10.4	
4.8	6.1					
6.1	6.5					
7.7	7.3					
4.6	4.4					
–	6.0					
–	6.1					
–	3.9					
–	7.7					
6.3		5.1				
6.3		3.9	6.3			
6.0			5.6			
8.7			7.6			
5.1				6.2		
Mean						
6.4 ± 0.3*	6.0 ± 0.5	4.5 ± 0.6	6.5 ± 0.6	--	9.5 ± 0.7	9.5 ± 0.7
Calculated lactic acid production, micrograms per milligram hour						
23.4 ± 1.2	24 ± 1.8	18.5 ± 2.5	26 ± 2.3	25	38 ± 2.8	38 ± 2.9
Lactic acid production, micrograms per milligram per hour						
23.9 ± 0.6	24.5 ± 2.5	26 ± 5.0	30 ± 4.6	28	42 ± 4.2	38 ± 1.2

*The numbers after the plus or minus signs are standard errors of the respective means. These are calculated by the formula S.E. $= \sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}$ in which x represents the successive variates in the sample, $\bar{x}$ the mean of the sample and n the number of variates.

the $Q_G^{N_2}$ of the uterus was about 35 per cent higher than in similar tissue from spayed controls, the difference tending to be greater during the first few hours of the experiments than later. However, the $Q_G^{N_2}$ of uteri treated in vitro with a number of concentrations of estrone (Table I) was not significantly different from that of untreated uteri, even at the end of six hours.

Decrease of sensitivity of the uterine cells to estrone during the fifteen to thirty minutes necessary for dissection and preparation of tissue probably does not account for the absence of effect of estrone in vitro, since injection of 0.1 and 0.01 microgram directly into the uterus before dissection was also ineffective.

It was thought possible that the failure of estrone to increase the $Q_G^{N_2}$ in vitro was caused by the absence of some necessary supplementary factor. A number of substances, succinate, malate, indole-acetic acid and standard chorionic gonadotropin, were added in the presence of estrone (Table II), but no such effect could be observed. If a supplementary substance exists, it was thought possible that this might be present in rat plasma. No evidence for

TABLE II

THE EFFECT OF SUBSTANCES OTHER THAN ESTRONE WITH AND WITHOUT ESTRONE ADDED IN VITRO ON $Q_G^{N_2}$ OF UTERINE TISSUE OF SPAYED RATS

CONTROL UTERI			UTERI WITH OTHER SUBSTANCES			
WITHOUT ESTRONE	WITH ESTRONE		SUBSTANCE	CONCENTRATION	WITHOUT ESTRONE	WITH ESTRONE
$Q_G^{N_2}$	$Q_G^{N_2}$	CONCENTRATION, MICROGRAMS, PER C.C.			$Q_G^{N_2}$	$Q_G^{N_2}$
3.4	3.9	5	Succinate	.0001 M	6.1	5.8
4.8	6.1	5		.0001 M	8.0	7.2
7.0, 7.3	–	0.01	Malate	.001 M	9.1	9.2
6.1	6.5	5	Indole-3-acetic acid	10/mg./1	6.7	5.2
7.4			Standard chorionic gonadotropin	10 I.U./c.c.	6.4	
6.6	–	5		10 I.U./c.c.	6.0	6.1, 5.7
6.3	3.9, 5.1	0.1		10 I.U./c.c.	–	3.4
8.7	7.6	0.01		0.1 I.U./c.c.	2.6	4.3, 6.1
6.0	5.6	0.01		0.1 I.U./c.c.	4.9	7.5
5.1	6.2	0.001		0.1 I.U./c.c.	7.0	6.6
7.4				0.01 I.U./c.c.	8.0	
6.3	5.1, 3.9	0.1		0.01 I.U./c.c.	–	2.8
7.4				0.001 I.U./c.c.	6.4	
		0.01	Plasma of uninjected rats	3 c.c.	8.0	6.8
		0.01			13.1	7.7
		0.01	Plasma of rats injected with 5 micrograms estrone	3 c.c.	8.4	9.1
		0.01	Plasma of rats injected with 50 micrograms estrone	3 c.c.	11.0	8.6

the presence of such a factor in plasma from rats in which estrone had not been injected or from rats into which large doses of estrone had been injected twenty-four hours before was obtained.

Carroll[3, 4] found that, when tissues were weighed at the end of an experiment, considerable error could be introduced through loss of particles of tissue into the medium. However, he used much smaller amounts of tissue than those used in these studies. If the absolute values for $Q\,\frac{N_2}{G}$ are perhaps too high because of this error, there proved to be no difference in the percentage dry weight between treated and control uteri, 18.8 per cent as compared with 18.9 per cent.

The rate of anaerobic respiration of uterine tissue either with or without estrone showed great variability. This would seem to be inherent in the tissue rather than in the method of measurement since the determinations of lactic acid agreed fairly well with manometric readings. Small variation of experimental conditions may be at fault, also. This large variation possibly may obscure a small effect of estrone in vitro, in no way comparable to the large increase of the rate of production of lactic acid in uteri from animals given estrone by injection.

COMMENT

Since estrone in any concentration used failed to increase the $Q\,\frac{N_2}{G}$ of uterine tissue when treated in vitro, it seemed likely that the increase of $Q\,\frac{N_2}{G}$ which was observed after injection of estrone into the whole animal either must depend on the presence in the tissues of some accessory substance brought there as a result of the injected estrone or must be due to a change of the nature of estrone itself or of the uterine cells which does not occur in vitro and probably requires time. That accessory substances necessary for action of estrone on the uterus are not present in the plasma of uninjected or injected rats was shown. Furthermore, experiments by Emmens[7] which show that estrone in minute amounts produces estrus in spayed animals when injected directly into the vagina, suggest that it is not necessary for estrone to enter the circulation to bring about its effects. This makes the necessity for the release of accessory substances from tissues other than the uterus itself unlikely.

It would seem, then, that either estrone as such is inactive on uterine tissue or that the changes of the rate of anaerobic glycolysis observed after injection of estrone are the result of some other change or series of changes in the cells of the uterus and cannot be assigned a primary role in the mechanism of action of estrogen.

SUMMARY

A series of concentrations of estrone in bicarbonate Ringer's solution was found not to have any effect on the rate of anaerobic glycolysis of uterine tissue of spayed rats in vitro. The lack of effect of estrone was not changed by the presence of a number of different substances.

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STUDIES ON THE INGESTION OF LARGE QUANTITIES OF PROTEIN AND AMINO ACIDS

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THE role of proteins in the diet has been appreciated and stressed for a long period of time. Rubner[1] some forty years ago pointed out that man cannot live on lean meat alone because of the limitation of the apparatus of mastication. This same idea is maintained in current textbooks of physiology[2, 3] which suggest that the limitations are due to those of digestion and absorption. It is appreciated that certain Eskimo tribes may subsist for long periods on diets of meat and fish, but in these foods the fat component is relatively high. Recently the effect of large quantities of protein in the digestive tract has received attention by reason of the fact that following massive gastrointestinal hemorrhage there is a considerable increase in the urea nitrogen content of the blood.[4-6]

The question of the superiority of amino acid mixtures over protein in the dietary management of certain pathologic conditions has attracted considerable attention. There is some feeling that orally administered amino acid mixtures might be superior to similar quantities of dietary protein,[7, 8] although the actual demonstration of such a superiority has not been made.

The present report describes studies carried out in two normal human subjects in whom comparable massive amounts of whole blood proteins, muscle proteins and amino acid mixtures were ingested orally. One objective of these studies was to compare the efficiency of the gastrointestinal tract in handling protein and in handling amino acids. A second objective was to get a better understanding of the effect of massive amounts of digested protein on blood and urine urea and blood amino acids. It was felt that the effect of massive gastrointestinal hemorrhage on blood urea could better be understood and evaluated with such information available.

METHODS

The experiments reported here consisted essentially of studying the changes in blood and urine during and following the ingestion of large amounts of meat, blood, and amino acids. The amount of nitrogen consumed in each case was of the same order of magnitude. This quantity represented the maximum

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amount of meat that could be voluntarily consumed over a period of approximately eight hours. The blood and amino acids were ingested over a comparable time interval. The meat was ground lean horse meat and was baked without addition of any other materials. The fresh whole beef blood was obtained from a local packing house and was immediately defibrinated and chilled. In this form it was quite palatable and was ingested in the same manner as one would drink milk. The solution of amino acids represented a casein hydrolysate fortified with tryptophane.* This solution was taken orally in the same way as the blood. The amount of nitrogen in each of the materials was determined by chemical analysis. The authors served as the experimental subjects. Their weights were 80 kg. (F) and 75 kg. (L). The meals on the day before each experiment were chosen so as to contain a minimum of protein, and in addition no food was consumed for eighteen hours prior to, and eighteen hours following, the experiment. The experiments were conducted about two weeks apart.

A blood sample was obtained in the fasting state and at 1, 3, 5, 8, 11, 16, 24, 33, and 48 hours after the beginning of the experiment. The blood was allowed to clot and the serum was analyzed for amino acid nitrogen, urea, and protein. A control urine sample was obtained for a three-hour period before starting the ingestion of the food materials and at frequent intervals for 48 hours thereafter. Urine samples were analyzed for urea, and the maximum urea clearance was calculated for each interval. The determination of urea in blood and urine was carried out by the colorimetric method described by Ormsby.[9] Serum amino acid nitrogen was determined by the colorimetric method of Folin as modified by Danielson.[10] This method was adapted to the use of the Evelyn photoelectric colorimeter by simple dilution of the final color. Plasma protein and all total nitrogen determinations were carried out by micro-Kjeldahl distillation procedures.

RESULTS

The maximum amounts of protein which each subject could ingest as ground baked meat were 480 grams (F) and 320 grams (L). This amount of food, representing $5\frac{1}{3}$ and $3\frac{2}{3}$ pounds of fresh meat, respectively, ingested over eight hours produced some gastrointestinal discomfort characterized by a sense of abdominal fullness. One subject (F) was able to ingest the same amount of protein in the form of blood (2,250 c.c. of whole blood) although the general abdominal discomfort produced by the blood exceeded that obtained with the meat. The second subject was able to ingest only 85 per cent as much protein in the form of blood as in the form of meat over the same time interval. Both subjects readily agreed that the amino acid mixture was much more disagreeable than either the meat or blood. One subject (L) had a severe attack of vomiting midway in this experiment so that total intake of amino acids was about half that of meat protein. The other subject was able to ingest a quantity of amino acid nitrogen equal to that ingested as blood or meat, but regards the experience as an extremely disagreeable ordeal. The gastrointestinal distress resulting in both subjects from amino acid ingestion

*Frederick Stearns & Co., Detroit, Mich.

was not due to the taste of this mixture which many persons do not like. A slight amount of diarrhea was obtained in one of the subjects (L) following blood and amino acids, but not in the other subject although prevention of a bowel movement required considerable voluntary control. A considerable diuresis was obtained in all of the experiments.

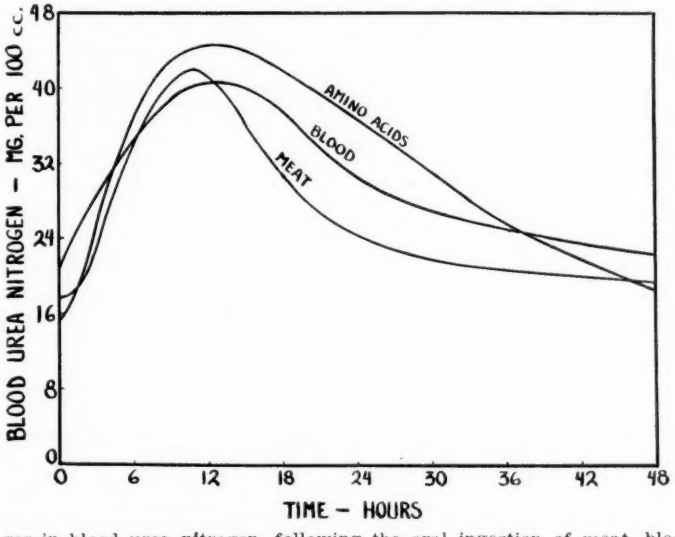

Fig. 1.—Changes in blood urea nitrogen, following the oral ingestion of meat, blood, or amino acids. Data on subject F who ingested 480 grams of protein or amino acid mixture.

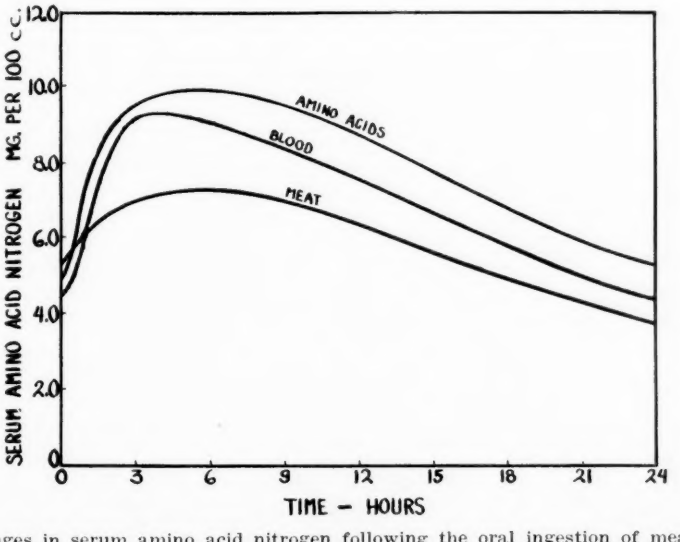

Fig. 2.—Changes in serum amino acid nitrogen following the oral ingestion of meat, blood, or amino acids. Data on subject F who ingested 480 grams of protein or amino acid mixture.

The changes in blood urea nitrogen and blood amino acid nitrogen for subject F obtained following the ingestion of blood, meat, and amino acids are indicated in Figs. 1 and 2. It will be seen that a comparable increase in blood amino acid nitrogen occurred following the ingestion of each of the three test substances although there was a lesser rise with the meat. The changes in

blood urea nitrogen were also comparable following the ingestion of blood, meat, or amino acids, with the amino acid mixture causing a slightly greater increase in blood urea nitrogen. The urinary urea nitrogen excretion in this subject is indicated in Table I. The ingestion of large amounts of proteins or amino acids caused a great increase in urea output in the urine. Calculations of the urea clearance indicate that there was a considerable increase in urea clearance during the height of the urea excretion in all three experiments. The total urea nitrogen excretion for forty-eight hours following the ingestion of the 76.8 Gm. of nitrogen in the form of meat, blood, and amino acids was 59.4 Gm., 72.1 Gm., and 71.1 Gm., respectively. Thus it appears as though most of the nitrogen derived from the proteins ingested was excreted as urea in the first forty-eight hours.

TABLE I

UREA EXCRETION AND UREA CLEARANCES FOLLOWING THE ORAL INGESTION OF MEAT, BLOOD, OR AMINO ACIDS

TIME INTERVAL HOURS	URINE UREA NITROGEN EXCRETION GRAMS PER HOUR			UREA CLEARANCE C.C. PER MINUTE		
	MEAT	BLOOD	AMINO ACIDS	MEAT	BLOOD	AMINO ACIDS
−3 − 0	0.78	0.93	0.72	77	73	79
0 − 2	0.66	1.05	0.62	65	75	59
2 − 4	0.80	1.25	0.94	62	76	61
4 − 8	1.56	2.20	1.43	81	112	69
8 −11	2.30	2.18	2.43	97	103	95
11 −16	2.17	2.35	2.62	92	105	100
16 −24	1.47	1.70	1.99	75	82	83
24 −33	0.92	1.18	1.27	60	70	67
33 −48	0.78	1.09	0.97	64	74	76

The plasma proteins were determined at intervals following the ingestion of amino acids. The values obtained were 6.8, 7.0, 6.9 and 7.1 Gm. per 100 c.c. at 0, 11, 33 and 48 hours, respectively. This does not represent any significant change. Electrophoretic studies of the serum proteins were also carried out to determine whether any of the protein fractions were changed. No changes were found.*

The results of the studies obtained on the other subject (L) were similar to those that have been presented for subject (F). The changes in amino acid nitrogen and blood urea nitrogen level of the blood were almost identical in the two subjects for the three test substances, the meat giving a lesser rise in serum amino acid nitrogen in both subjects. The blood urea nitrogen increase following amino acid ingestion was somewhat less than that obtained in subject (F) undoubtedly due to the fact that only about one-half of the amount of the amino acid nitrogen was ingested. Maximum urea clearances were increased with all three test substances and almost all of the ingested nitrogen was excreted as urea nitrogen in the urine.

DISCUSSION

The authors agree with the statements made in texts of physiology which suggest that a man cannot readily meet his daily caloric requirement by the ingestion of lean meat. The fact that hydrolyzed amino acids cannot be in-

*The electrophoretic studies were carried out by Lt. C. L. SanClemente, to whom the authors are deeply grateful.

gested in quantities exceeding that of protein indicates that the limiting factor is not one of mastication as Rubner[1] originally suggested. Neither is it one of digestion, since the amino acids do not require digestion, but rather it is the rate of intestinal absorption that defines the maximum intake of protein or amino acids. The high osmotic pressure of the amino acid mixture undoubtedly greatly retards its gastric emptying. The authors have observed that a quantity of glycine as small as 50 Gm. is only very slowly emptied from the human stomach, 50 per cent of this quantity remaining at the end of three hours. In three subjects this amount of glycine caused considerable nausea and gastrointestinal distress of the same nature as that obtained with the amino acid mixture.

The blood urea clearance figures indicate that there is an actual increase in the maximum clearance as well as in the amount of urea excreted. These results are in accordance with the observations of Jolliffe and Smith[11] on dogs and Longley and Miller[12] in human beings, in which it was found that high levels of protein intake elevated urea clearance values. Study of the data in Table I indicates that the rate of urea excretion is from 2.5 to 3.6 times as great during the time that the blood urea level is increased. This great increase in the rate of excretion of urea by the normal kidney is quite important in minimizing the extent of blood urea elevation. In an individual with some impairment of renal function it is apparent that, if the kidney could not increase its rate of urea excretion, the blood urea level would show a much greater rise. The same effect would tend to be observed following massive gastrointestinal hemorrhage where the functional efficiency of the kidney is decreased as a result of diminished blood pressure. The mechanism of the rise in blood urea following digestion of large amounts of protein or following severe hemorrhage is undoubtedly different from that observed by Myers[13] in patients with chronic peptic ulcer.

The value for the blood amino acid nitrogen levels, for the blood urea nitrogen levels, and especially for the urea excretion and the maximum amount of the substance that could voluntarily be ingested indicate that the over-all processes of digestion, absorption, and metabolism of muscle proteins as compared with blood proteins are approximately the same. Furthermore the data obtained following the ingestion of the amino acid mixture provide further evidence that the controlling or limiting factor is the rate of intestinal absorption. These results raise a definite question as to the efficacy of orally administered amino acids and their possible superiority over dietary protein. If there is an inadequacy of pancreatic proteinases to accomplish the hydrolysis of dietary protein, then orally administered amino acids might be of advantage. On the other hand, the osmotic pressure of amino acid mixtures is many times as great as that of protein and for this reason they have an irritating effect on the gastrointestinal tract. From the experience with normal subjects it would appear that under almost all circumstances protein would be more readily tolerated by the gastrointestinal tract than amino acids. Obviously the utility of intravenously administered amino acids is in no way concerned in this discussion.

One purpose of this investigation was to determine whether or not the azotemia following extensive gastrointestinal hemorrhage may be used to

evaluate the extent and duration of the bleeding. Attempts have been made to utilize the blood urea nitrogen levels as diagnostic and prognostic aids.[6, 14-16] The value of such attempts has also been disputed.[17] In one experiment described in this report 76 Gm. of protein nitrogen in the form of blood were ingested, but never as much as 3 per cent of this nitrogen appeared in the blood at any one time as increased blood urea nitrogen. If one assumes equal distribution of urea in all of the body water the quantity of increased urea nitrogen is always less than 20 per cent of the nitrogen ingested. It is readily apparent that the increase in blood urea nitrogen is influenced by factors other than the amount of blood in the gastrointestinal tract. These may include differences in the rates of digestion, absorption and metabolism of protein as well as differences in renal efficiency in different normal subjects. The latter factor would be even more important in patients following extensive hemorrhage due to variable decreases in blood pressure. Thus it would appear as though the measurement of the blood urea nitrogen level is an extremely crude method of ascertaining the extent of gastrointestinal bleeding, since the extent of elevation is a function both of the amount of protein digested and of the rate of urea excretion. The latter factor is subject to wide variations.

The data on urine urea nitrogen excretion in Table I indicate that the urea excretion is a good index of the amount of protein metabolized, particularly if some attention is also directed toward blood urea levels. It is readily admitted that any blood protein that is lost by vomiting or by diarrhea will not be detected by studies of the urea content of urine. The extent of hemorrhage, however, will be much more accurately evaluated by urine and blood studies than by blood studies alone. Sufficient information can be obtained if two complete 24-hour urine collections are made and the total urea nitrogen is determined. This figure multiplied by the factor 6.25 will give an index of the amount of protein metabolized, particularly if it is known that the blood urea has not changed greatly during this period. Corrections for any increase in blood urea nitrogen are readily calculated assuming that urea is equally distributed in all of the body water. From the amount of protein metabolized it is possible to calculate the amount of blood that would have to be digested to supply this quantity of protein. Deductions should be made for any dietary protein ingested during the period. It would appear that this method, although subject to certain limitations, gives a more reliable estimate of the extent of a gastrointestinal hemorrhage than any of the methods in use at the present time.

SUMMARY AND CONCLUSIONS

1. Maximum amounts of blood, muscle meat, and amino acid mixture were ingested over an eight-hour period by two normal human subjects. Studies of blood urea, blood amino acids, urinary urea excretion, and urea clearances were carried out.

2. The rate of intestinal absorption appears to be the limiting factor in determining the amount of protein or amino acid mixture that can be ingested. The normal gastrointestinal tract more readily handles large amounts of unhydrolyzed protein, than it does equivalent amounts of amino acids. This raises a question as to whether amino acids have any advantage in alimentation

over unhydrolyzed dietary protein, especially when relatively large amounts are administered and when pancreatic proteinases are present in adequate amounts.

3. The variations in the blood levels of urea nitrogen and amino acid nitrogen were similar after the ingestion of the two proteins and the amino acid mixture.

4. Maximum urea clearances, as well as urinary urea excretion, were increased following the consumption of amino acids and proteins.

5. The extent of a gastrointestinal hemorrhage can be more accurately evaluated by urine and blood urea determinations than by blood studies alone.

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LABORATORY METHODS

GENERAL

THE SIGNIFICANCE OF THE pH IN THE GOLD REACTION

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THE satisfactory performance of tests that represent modifications of the original gold reaction[1] requires an understanding of the effect of the pH of the milieu on the character of the curves. If the pH is outside the relatively narrow optimum range, the results of tests will be vitiated. At the time that Zsigmondy published his work on the so-called "gold number,"[2] and when the original paper concerning the gold reaction appeared in 1912, the pH was not then taken into consideration. No trouble attributable to the pH, however, was encountered so long as the original technique employing Zsigmondy's formol gold was followed, because, fortunately, the pH happened to be in the optimum range. Early modifications aimed at technical simplification involved replacement of the formol gold, since the preparation was claimed to be too difficult for clinical laboratories. This difficulty, encountered by many laboratory workers in the preparation of formol gold, was actually the only safeguard for the maintenance of an adequate pH of the milieu in the original gold reaction, as batches of colloidal gold satisfactory in appearance were certain to be properly adjusted with regard to the pH. When the formol gold was replaced by modifications, these safeguards were lost.

Now that the significance of the pH in the gold reaction is understood the use of gold sols of varying pH is possible, provided the milieu in which the reaction takes place is properly adjusted. The variations in the pH of the three reagents taking part in the reaction—cerebrospinal fluid, diluent, and gold sol—and the effect on the pH of the milieu will be analyzed first.

THE pH OF EACH OF THE THREE REAGENTS

The pH of freshly collected cerebrospinal fluid is 7.4, that is, almost neutral. Unless loss of carbon dioxide is prevented, the pH rises markedly in a few hours. The pH of mailed cerebrospinal fluid is usually about 8.4 to 8.6, a reaction sufficiently alkaline to disturb the pH of the milieu.

The pH of the diluent is of far greater importance, since this reagent can be made to stabilize the reaction in the optimum range. In the original gold reaction, 0.4 per cent saline was used in preparing the serial dilutions of cere-

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brospinal fluid. The pH of saline is about 5.7 to 6.0, a markedly acid reaction, due to absorption of carbon dioxide from the air. The pH of serum diluted with saline remains unchanged in the lower dilutions, since the highly concentrated protein acts as a buffer, while in higher dilutions the low pH of the saline exerts an effect. On the other hand, this buffering effect is negligible in cerebrospinal fluids normally containing only traces of protein and the pH may vary in serial dilutions from 8.6 to 5.7.

Reducing agents other than formol have been used in the preparation of gold sols, and have yielded hydrogen-ion concentrations differing markedly from the gold sols prepared with formol. Since the reduction with sodium citrate introduced by Borowskaja[3] is the most satisfactory, discussion will be confined to this type. The sensitivity and appearance of citrate gold sols vary widely, depending on the concentration of the alkaline sodium citrate used to reduce the acid gold chloride. Stable gold sols can be prepared with sodium citrate concentrations varying from 1:1,000 to 1:15,000. With decreasing concentrations of the alkaline sodium citrate there is, of course, a decrease in the pH, while the sensitivity of the gold sol increases. There is no sharply restricted optimum pH for the reduction. The reaction, however, must be acid, since no reduction occurs at or above the neutral point. Borowskaja used a high concentration of the sodium citrate, namely 1:2,000. The resulting gold sol, which for convenience may be called "citrate gold 1:2,000," is extremely "hard," that is, insensitive. The pH is about 6.0 by potentiometric determination. Saline is used as a diluent.

In the quantitative gold reaction, of which a detailed description has been given in a previous publication,[4] "citrate gold 1:10,000" is used, the pH of which is, of course, even lower, namely 4.8 to 5.0. This "softer" citrate gold, however, exhibits the same optimal sensitivity as formol gold. The marked acidity is of no consequence, because the final pH of the milieu is regulated by buffers.

The optimal sensitivity of gold sols is directly dependent not only on the dispersity of the sol itself but also on the pH of the milieu. If a gold sol that is too hard, such as that of Borowskaja, is used in a milieu of correct pH, that is, 7.4, the results are inferior because the sol is too insensitive. If, on the other hand, the sensitivity of a gold sol that is too hard is raised by a pH of the milieu that is too low, the results of the gold reaction may be rendered definitely false, as indicated by the occurrence of false paretic curves subsequently discussed. In short, only through a combination of an optimal sensitivity and an optimum pH of the milieu can best results be reproduced.

EXPERIMENTAL DETERMINATION OF THE OPTIMAL pH OF THE MILIEU

The optimal pH of the milieu depends to some extent on the sensitivity of the gold sol. It is determined in the following experiments on the basis of a sensitivity believed to be optimal, and that, in addition, can be reproduced without difficulty. The technique of the quantitative gold reaction[4] is employed. The term quantitative is applied since the principle of the reaction involves the use of a gold sol of constant and optimal sensitivity and the maintenance of a constant and optimal pH of the milieu through strong phosphate buffers. In

TABLE I

EFFECT OF INADEQUATE pH ON RESULTS OF GOLD REACTION

NO.	MATERIAL	TOTAL PROTEIN MICRO-KJELDAHL MG. %	PHOSPHATE BUFFERS pH	GOLD REACTION—SERIAL DILUTIONS*										SUM TOTAL†	TYPE OF GOLD CURVE
				1:15	1:23	1:34	1:51	1:76	1:114	1:171	1:256	1:384	1:576		
1	Purified sheep hemoglobin solution	4340	7.2	0	0	0	0	0	2	2	3	1.5	1.5	10	
2			7.0	20	20	20	18	18	17	17	17	15	9	171	
3	Normal cerebrospinal fluid	19.4	7.4	2.5	3	3.5	∧ 4.5	4	3.5	3	2.5	1.5	1	29	Typical normal
4	The same normal cerebrospinal fluid to which was added 1:100 volume of hemolyzed human blood	207	7.4	2	2.5	2	2	2	2	2	2	2	2	21	Atypical curve in subnormal range
5			6.5	18	18	18	18	18	18	18	18	18	18	180	Strong paretic
6	Cerebrospinal fluid from meningococcus meningitis	108	7.4	3	3	3.5	4	5	6	8	9	∧ 11	8.5	61	"Meningitic"‡
7			6.2	18	18	18	18	17	17	16	16	16	16	170	Atypical paretic

*Note changed serial dilutions, begin with 1:15, common factor ⅔, instead of ½.

†Sum of the 10 color values read with color standard; expresses strength of reaction. Values range from 0 red to 10 blue, to 20 colorless. ∧ marks the maximum reaction obtained by reading after two hours.

‡"Meningitic" now called "hematogenous type" indicating the passage of blood colloids into the cerebrospinal fluid.

these experiments presented in Table I, buffers of varying pH but of uniform molecular strength were used.

Citrate gold 1:10,000 was employed because through its use the reproduction of correct gold sols was remarkably easy. In order to eliminate the deleterious effect of aging, use of this gold sol was restricted to a period of one week. There were no disadvantages in following this precaution, because the gold sol is not only easily prepared, but is exceedingly inexpensive. This technique, which can be readily adopted in any competent laboratory, was followed in these experiments (see Table I). The results were read with the help of an easily prepared gold color standard and were in this way objectively recorded.

A few readily duplicated experiments suffice to demonstrate the detrimental effect of an excessively low pH on the results of the gold reaction. Experiments 1 and 2 show the striking effect of such a slight change of the pH as from 7.2 to 7.0, on the reaction between gold sols and purified hemoglobin solution, the isoelectric point of which lies at about 6.8 to 7.0. No. 3 demonstrates the reaction of a normal cerebrospinal fluid in the quantitative gold reaction, while Nos. 4 and 5 show the reaction of the same cerebrospinal fluid after the addition of 1:100 volume of hemolyzed human blood. When the milieu is adjusted to pH 7.4, the blood has a weakening effect, while at pH 6.5 a fundamental change in the character of the curve appears; No. 5 is an example of a "false paretic curve." It exactly counterfeits a true paretic curve, but it is technically false, and can be easily avoided by the maintenance of an adequate pH. This is of great practical importance in the examination of fluids containing small amounts of blood that have been accidentally introduced during the tap and have become hemolyzed. No. 6 exemplifies the typical "shift to the right" or so-called "meningitic curve" occurring at a pH of 7.4 with cerebrospinal fluid from a case of meningitis. No. 7 shows the effect of a low pH, 6.2, on the same cerebrospinal fluid. In this case, even in the absence of hemoglobin with its high isoelectric point, occurrence of the most dangerous type of false reaction is demonstrated. It is obvious that the value of a paretic curve becomes nil if, of all types of the gold reaction, this most important one is polluted with a considerable percentage of technically false paretic curves. The appearance of paretic curves in purulent meningitis constitutes the acme among false reactions since, of the protein patterns indicated by the distinguishable types of the gold reaction, those encountered in paresis and in purulent meningitis exhibit in every respect the maximal difference.

It has thus been demonstrated that false paretic curves may be elicited by a pH that is too low. The higher the pH of the milieu, the greater the safeguard against "false paretic curves." Above pH 7.4, however, the strength of the reaction decreases unfavorably. Accordingly, pH 7.4 was selected as optimum. It is safely above pH 7.0, the danger zone in which hemoglobin may render the results of the colloidal gold reaction entirely misleading. It is noteworthy that this pH, 7.4, is optimum for the reduction of Zsigmondy's formol gold sol.

DISCUSSION

The pH of the milieu has been shown to be of great importance. The pH of the cerebrospinal fluid and gold sol is significant only in systems in which the

pH of the milieu is not adjusted, as is the case when saline is used as a diluent. When strong buffers are employed, the pH can be fixed at the optimum value, regardless of the particular pH values of the cerebrospinal fluid and gold sol. Modifications of the original gold reaction that do not include a satisfactory regulation of the pH can be shown to yield erroneous results. The obtaining of a so-called paretic type of curve in the examination of a specimen from a case of purulent meningitis is evidence that the technique employed is worse than valueless. Experiments described in this paper indicate that such results may be elicited by a pH that is too low, and experience has shown that they occur only when the pH is unsatisfactory. In more than thirty years, using at first the original gold reaction, which is only a qualitative test, and later the quantitative gold reaction,[4] the senior author has never observed anything suggesting a paretic type of curve in cases of purulent meningitis. The regulatory mechanisms governing the pH in these two techniques have been explained. The pH of the milieu in the case of the original gold reaction was not fixed and constant, since buffers were not used; however, the pH usually remained in a range high enough to assure good results. When too low, the appearance of the sol was obviously wrong. The quantitative gold reaction requires buffers to maintain a constant pH at the optimum level. The pH in modifications of the gold reaction in which it is not taken into consideration is usually incorrect, either owing to the use of gold sols that are themselves acid, or to the acidifying of gold sols in order to increase their sensitivity to a satisfactory level.[5] The combination of inherently acid gold sols and a saline diluent leads to erroneous results; the magnitude of error is restricted, however, since the fall in pH is limited. Error in the highest degree may be encountered in all modifications in which acid is added to gold sols in order to increase their sensitivity; under such circumstances the pH of the milieu is certain to be inadmissibly low. While inherently acid gold sols may yield optimal results in combination with buffers that insure the correct pH; buffers cannot be used for this purpose when gold sols are acidified according to the Krebs principle, since adequate sensitivity of the inadequate gold sol is obtained only by adjusting the reaction at a pH far below the optimum for the test itself. The fact that satisfactory results cannot be obtained under such circumstances is convincingly demonstrated by the occurrence of "false paretic curves."

SUMMARY

Optimal and reproducible results of the gold reaction depend to a large extent on a constant and optimal pH of the milieu. The alkalinity of cerebrospinal fluids or the acidity of gold sols can affect the pH of the milieu unfavorably only if saline is used as a diluent. Strong buffers are superior in every respect because they serve to maintain a uniform and correct pH. The optimal pH of the gold reaction is 7.4, provided that an easily prepared citrate gold sol of constant and optimal sensitivity is used. A pH of the milieu that is too low causes erroneous results. An inadmissibly low pH may easily be detected in unsatisfactory modifications of the gold reaction by the appearance of false paretic curves in purulent meningitis.

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A RAPID AGGLUTINATION TEST FOR THE DIAGNOSIS OF TULAREMIA

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THE diagnostic laboratory, in setting up routine agglutination tests, is not infrequently requested to include in the series the *Pasteurella tularensis* antigen. The results of such tests are generally read and reported after an incubation period of 18 to 24 hours, although it would be an advantage in many cases if the report to the physician could be made earlier.

In our experience it has been observed that definitive results with the *P. tularensis* antigen can be obtained almost immediately. This is accomplished by setting up the routine series of serum dilutions—in this laboratory 1/40, 1/80, 1/160, and 1/320—and adding the same antigen used in the test tube agglutination. The tubes are then shaken vigorously, by hand or in a Kahn shaker, for four or five minutes and read.

For the sake of comparison we have recorded the immediate results of the "shake test" and parallel incubated tests after the usual 18 to 24 hours. Thus far the degree of reaction observed has been identical, not only in the routine dilutions but in the final titer of the serum, except in a few instances where a slightly stronger reading has been obtained following the overnight incubation.

TABLE I

P. TULARENSIS AGGLUTINATION OBTAINED BY THE RAPID AND CONVENTIONAL METHODS

SPEC. NO.	SHAKE TEST				TITER	WATER BATH TEST				TITER
	1/40	1/80	1/160	1/320		1/46	1/80	1/160	1/320	
75	4+	4+	4+	4+	4+ 1/320	4+	4+	4+	4+	4+ 1/320
106	4+	4+	4+	4+	4+ 1/2560	4+	4+	4+	4+	4+ 1/2560
162	4+	4+	4+	4+	4+ 1/2560	4+	4+	4+	4+	4+ 1/2560
279	4+	4+	4+	4+	4+ 1/2560	4+	4+	4+	4+	4+ 1/2560
499	3+	4+	4+	4+	Not determined	4+	4+	4+	4+	Not determined
585	3+	2+	–	–		4+	2+	–	–	
3436	4+	3+	1+	–		4+	3+	–	–	
C. C.	4+	4+	4+	4+	4+ 1/640	4+	4+	4+	4+	4+ 1/640
R. G.	4+	4+	4+	4+	4+ 1/5120	4+	4+	4+	4+	4+ 1/5120
Dog 1644	4+	4+	4+	4+	4+ 1/1280	4+	4+	4+	4+	4+ 1/1280
S.S.	4+	4+	4+	4+	4+ 1/2560	4+	4+	4+	4+	4+ 1/2560

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e.g., a 3+ has become 4+. In no instance has any degree of reaction been obtained in an overnight test when the results of the "shake test" were completely negative.

Table I gives typical results obtained by both procedures, together with the final titers of the sera. All the specimens were from human sources except one, which was from an experimentally infected dog.

CONCLUSION

Reliable agglutination results in suspected cases of tularemia may be obtained almost immediately by the technique described.

A SIMPLE INEXPENSIVE APPARATUS FOR THE STERILE LYOPHILIZATION OF SMALL AMOUNTS OF MATERIAL

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THE Department of Preventive Medicine and Public Health of the University of Texas has been conducting researches on Rickettsial diseases for the past four years; this work has required the use of a large number of laboratory animals. In the past, all strains of our passages were kept in guinea pigs and rats. Because of the expense involved, however, another method was sought for preserving these strains. It seemed desirable to try lyophilizing tissues, but we did not feel justified in purchasing a lyophilic apparatus. We developed, therefore, a setup capable of drying small amounts of material which is simple in design and construction; its cost is negligible.

The apparatus is made entirely of Pyrex glass so that cleaning and sterilization may be done with dry heat. The design decided upon requires only the simplest of glass blowing and offers no great difficulties in construction. With it, from 2 to 5 c.c. of material can be dried in three to five hours' time, depending on the amount of tissue left in suspension. The material to be dried is ground in a mortar; alundum is used whenever necessary. Sterile skimmed milk is added and the whole is thoroughly mixed. Two to five cubic centimeters of this mixture is put into the drying tube and placed in the lyophilic apparatus. When alundum is used, the material is centrifuged or strained through four thicknesses of gauze before it is put into tubes.

The apparatus is illustrated in Fig. 1. Its assembly requires (1) a 500 ml. round bottom flask with three 24/40 standard taper necks; (2) three 24/40 standard taper inner connections with one of them having an inner extension; (3) a Pyrex glass stopcock; (4) a 29/42 standard taper inner and outer connection; (5) a straight walled Pyrex vacuum bottle having a diameter of 15 cm. and a depth of 25 cm.; (6) a laboratory pump supplying adequate vacuum; and (7) a standard drying tower.

CONSTRUCTION

The inner 29/42 connection is sealed to one end of a piece of 25 mm. Pyrex glass tubing 20 cm. long, and the 24/40 connection with the inner extension is sealed to the other end. The inner connection is cut off 3 cm. from the ground glass joint so that it will project a short distance into the flask when the apparatus is assembled. The glass tubing is bent to form a 70 degree angle in such a way that there are no constrictions to obstruct the free flow of air. The outer 29/42 connection is then sealed off at its distal end so

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that it forms a cup 13 cm. deep. A 15 cm. test tube when placed in this cup extends a short distance up into the inner 29/42 connection when the two are joined.

A short piece of Pyrex glass tubing is then sealed onto another 24/40 inner connection and bent at a right angle to act as a connection to the vacuum pump. A Pyrex glass stopcock is sealed to the end of another 24/40 inner connection and serves as a means of attaching a manometer to the system and as a means of releasing the vacuum when so desired.

The connection to the pump is made through a short length of heavy walled rubber pressure tubing having an inner diameter no less than the 6 mm. opening of the vacuum pump. The connection to the manometer is also made with rubber pressure tubing of small bore.

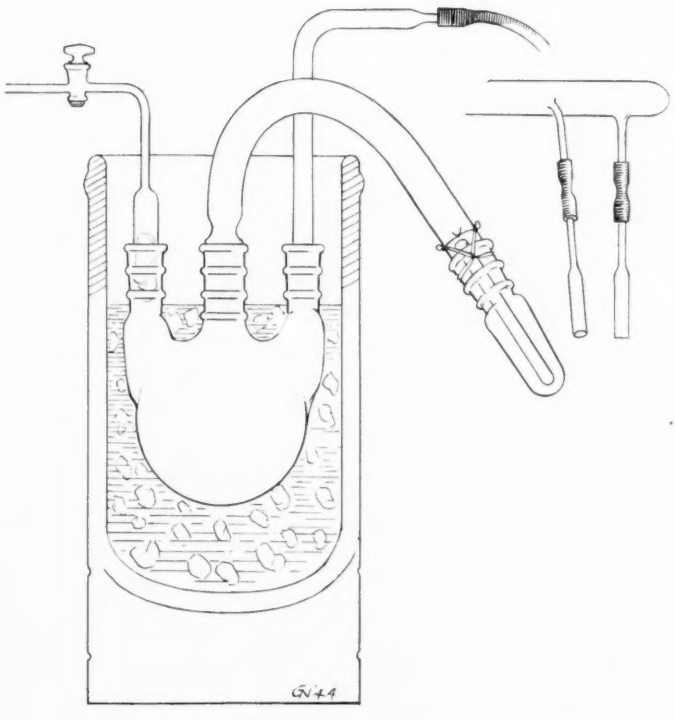

Fig. 1.

OPERATION

After the application of a thin layer of stopcock grease to all joints, the three 24/40 inner connections are put into place in the 500 ml. flask; the joint having the inner extension is placed in the center neck. The rubber connections are made and the flask is placed in the vacuum bottle and packed in dry ice. The material to be dried is placed in a 15 cm. test tube with a 12 mm. diameter and is frozen to the walls by being rotated at an angle in a dry ice-acetone freezing mixture. The tube is then placed at once in the cup formed by the 29/42 connection and attached to the apparatus. The pump is started and drying is begun.

After an adequate drying period, the pump is shut off and air which has been passed through both a drying tower packed with calcium sulfate and through a sterile cotton filter, is slowly allowed to enter the system through the stopcock. After the vacuum is released, the test tube is removed from the apparatus and closed immediately with a close fitting sterile rubber vial cap. A sterile needle, attached to the vacuum pump through a length of rubber tubing, is put through the rubber cap and the tube is again evacuated. The needle is rapidly withdrawn and the stoppered end of the tube is plunged at once into melted paraffin to seal any possible leaks.

Subsequent re-solution of the material in the same sterile container without contamination from outside air can be accomplished by simply injecting through the rubber stopper any desired amount of diluent. If small amounts of the dry material are removed, the rest may be preserved if a fresh sterile cap is used and evacuation is done as before.

The utility of the apparatus is increased if a second manifold, which allows the use of ampules, is made in the following way: A 20 cm. piece of 20 mm. Pyrex tubing is sealed to a 24/40 standard taper joint having an inner extension. The inner extension is cut off as before and a right angle bend is made in the tubing. The end of the tube is sealed off and two side arms of 8 mm. Pyrex tubing are sealed into the manifold to allow for the attachment of two ampules. The manifold is placed in the center neck of the 500 ml. flask and the ampules are attached by means of a short length of close fitting pressure tubing. After dehydration is complete, the ampules are sealed off by means of a gas-oxygen flame.

Since difficulty was experienced in sealing the ampules while evacuated, it was decided to fill the ampules with nitrogen gas. This was accomplished by filling a spirometer of sufficient volume with nitrogen and attaching the spirometer to the lyophilization apparatus by a length of rubber tubing leading to the stopcock in the 500 ml. flask. A layer of mineral oil on top of the water in the spirometer keeps the gas fairly dry. The stopcock is then opened slightly and the gas is slowly allowed to replace the vacuum. When the gas passes through the flask, any water present is removed. After the vacuum is neutralized, the ampules can be sealed without any difficulty.

SUMMARY

The construction and operation of an apparatus suitable for the sterile lyophilization of small amounts of material is described.

AN IMPROVED RESTRAINING DEVICE FOR HEMATOLOGICAL WORK WITH RATS AND MICE

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IN OUR laboratories considerable difficulty has been experienced in restraining small animals like rats and mice for routine blood examination. Various devices such as special boxes, glass test tubes, wrapping in towels, etc., have been used in the past. These methods have in general proved cumbersome or time consuming and some did not have sufficient flexibility to accommodate animals of different sizes.

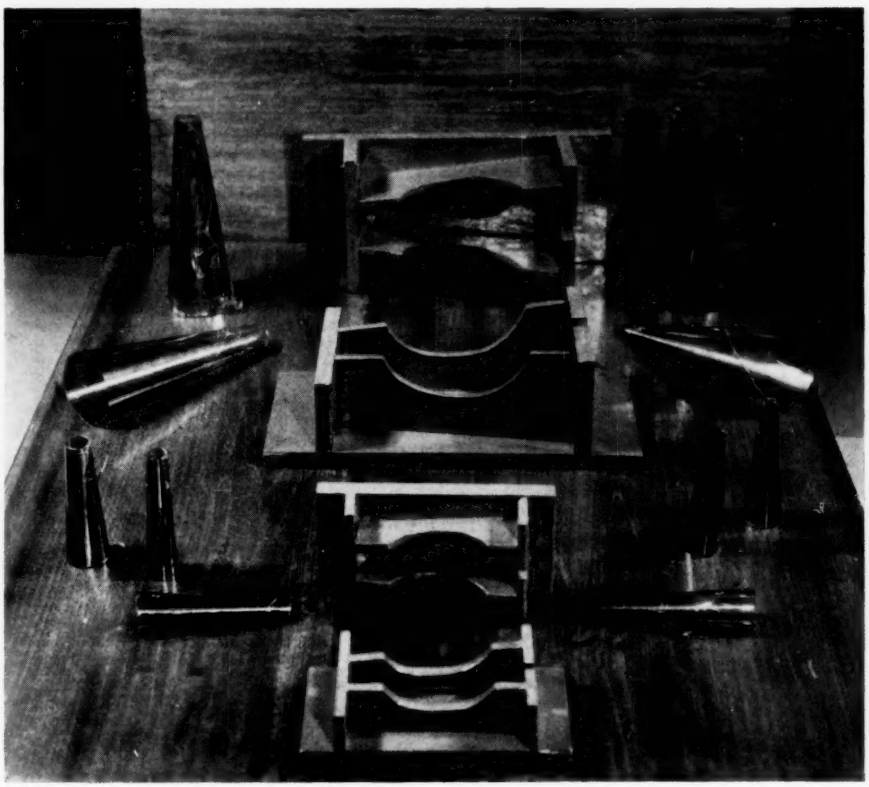

Fig. 1.—Two rat boxes and two mouse boxes are shown, as well as a number of cones of various sizes.

Because of the rather large number of rats and mice involved in our work, the necessity for an improved method of restraining the animals became acute. Therefore a number of cones of cellulose acetate (washed x-ray films) were wound on a tapered wooden form having the large diameter sufficient to ac-

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commodate the largest rat used, with the taper approximately the same as the average rat and the smaller open end of such a size that the animal's head could not emerge. A number of cones of various diameters were assembled to allow some selection for animals of different sizes. Transparent scotch tape proved to be more satisfactory for holding the cones together than did cements which were first tried. Experience soon showed that a length of about 7 inches was sufficient to hold any laboratory rat in our stock. A shallow, open topped

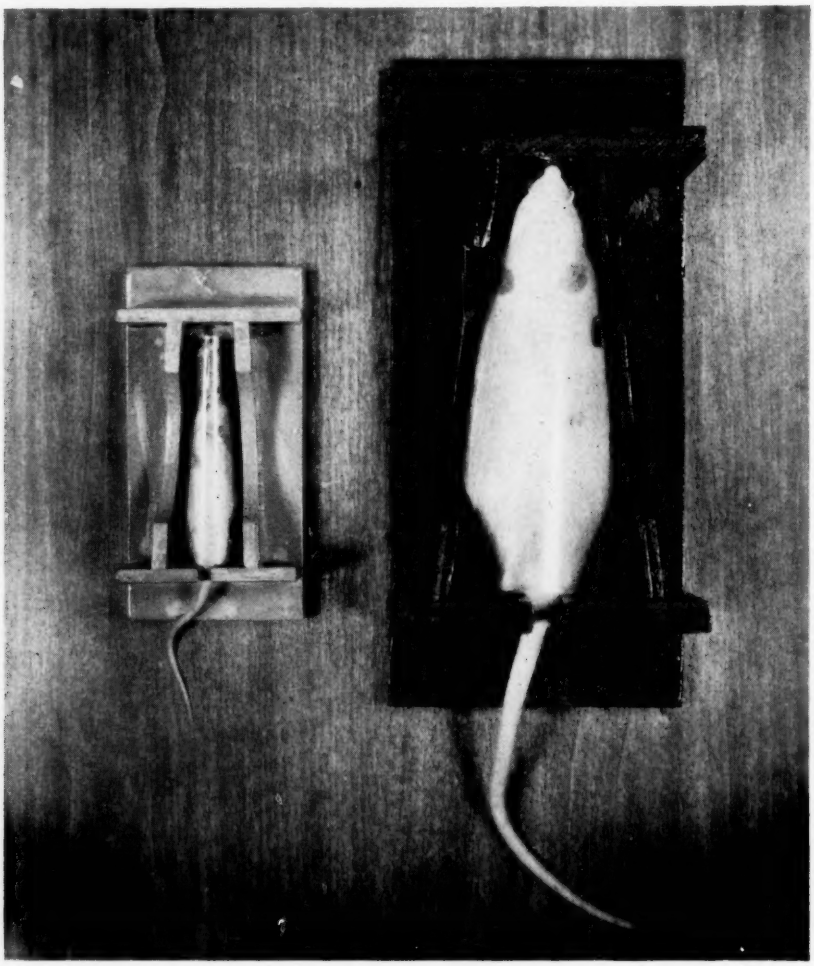

Fig. 2.—Rat and mouse boxes loaded for work.

wooden box (Fig. 1) was constructed with the sides set at the angle necessary to guide and support the plastic cones described above. Wooden cradles were tacked to the ends of the box on the inside to hold the cones more or less level. A slit was made on the back of the box to allow the tail of the animal to hang out (Fig. 2). The end of the tail could then be snipped with a scissors to obtain blood for counts, films, etc. The arrangement constructed for mice is similar except that the boxes and cones are built to a scale suitable to the smaller animal.

Most of the animals enter the cone willingly when it is presented; once they enter, the cone is tipped so that the animal has to climb. Then the loaded cone is placed in the wooden holder and counts are made. The boxes are covered with a waterproof paint so that they are easily cleaned with soap and water.

A simple holder has been described which greatly facilitates the handling of small animals such as rats and mice for hematological work. One great advantage is that the technician can perform the whole procedure without assistance.

A CANNULA FOR MEASURING INTRATHORACIC PRESSURE

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IT IS sometimes important to measure the intrathoracic pressure and follow the changes in its magnitude during both inspiration and expiration under various experimental conditions. The apparatus and method described below yield uniform results. The apparatus is simple and can be used continuously during an experiment. The cannula can be introduced repeatedly, because of its small caliber, without leaving a leak in the thoracic wall at the end of several observation periods. We have used it on dogs and obtained very satisfactory results.

The Y-shaped cannula, shown assembled in the top diagram of Fig. 1, consists of the following parts: a trocar in the form of a needle control valve (*a*), knurled packing control (*b*), rubber packing (*g*), housing for needle control valve (*c*), knurled adjustment nut (*d*), and a sponge rubber washer (*e*). The portion of the cannula that is introduced through the thoracic wall into the pleural cavity is about one inch in length and one-sixteenth of an inch in diameter. In the bottom diagram of Fig. 1, the sectional view of the cannula demonstrates its connection with the intrathoracic cavity at the black arrows. The white arrows demonstrate the continuity of the intrapleural space with the lumen of the apparatus and rubber tubing (*f*), which connects with a water manometer not shown in this diagram. The U-shaped water manometer to be used with this apparatus should have long arms in order to provide for ample excursions of negative and positive variations in intrathoracic pressure. The water in the manometer may be colored with a blue or red dye to facilitate accurate reading of the pressures.

The shaft of the cannula with the tip of the trocar in its lumen is introduced into the intrapleural cavity through an intercostal space, preferably close to the upper border of a rib near the middle of the lateral wall of the thorax. The depth can be regulated by the use of one or more sponge rubber washers (*e*), and by the knurled adjustment nut (*d*), both of which are anchored to the chest wall by four skin sutures as seen on the anterior view of the knurled nut. After introduction of the shaft of the cannula into the pleural space, the trocar is pulled out as far as the grooved mark, thus establishing an airtight communication between the thoracic cavity and the manometer. A T-connection should be placed in the tube leading to the manometer to provide a means for introducing 50 c.c. of air into the pleural space to facilitate registration of the intrathoracic pressure. Several lateral perforations are made in the part of the

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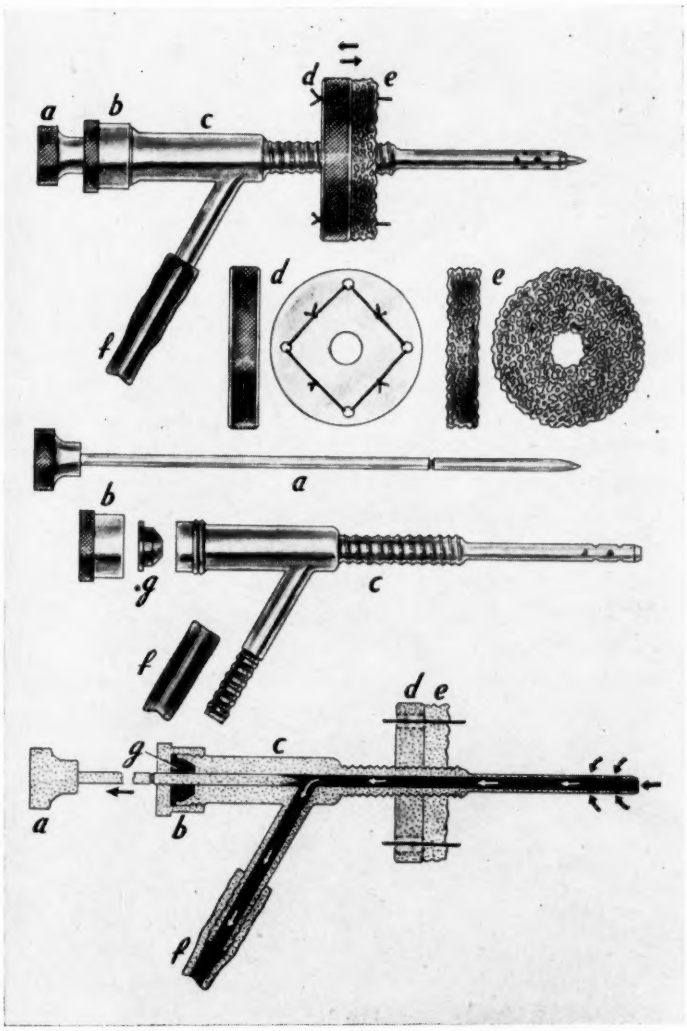

Fig. 1.—The top diagram shows the Y-shaped cannula assembled. The middle diagram shows the constituent parts, and the lower diagram gives a sectional view of the cannula when it is in operation for recording intrathoracic pressure. (Drawing made by J. F. Glore.)

cannula which is introduced into the pleural cavity in order to maintain continuity between the thoracic cavity and the manometer which otherwise might be interrupted by contact of the long surface with the terminal hole during the expansion of the lungs.

A MODIFIED PIPETTE FOR PROTHROMBIN DETERMINATIONS

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WE HAVE frequently encountered difficulty in pipetting small amounts of reagents into test tubes such as are used in the Quick method for prothrombin determination. If the tip of the pipette is placed near the top of the test tube, the reagent has to run down the length of the tube, with the result that a considerable part of the 0.1 c.c. of reagent used is lost. On the other hand, if the tip of the pipette is placed near the bottom of the test tube, there is a tendency for the reagent to form a film between the pipette and the test tube wall, again resulting in the loss of material.

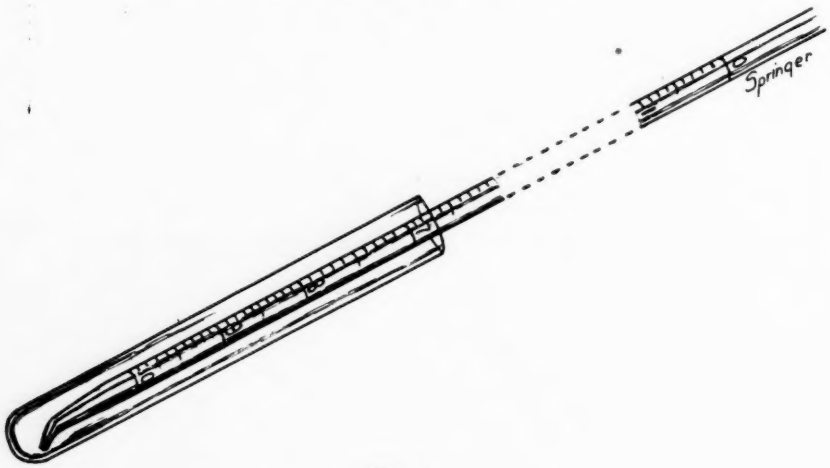

Fig. 1.

We have found that these difficulties may be overcome by drawing out and bending the tip of the pipette as shown in the diagram. With a pipette so modified, the tip may be placed near the bottom of the test tube without the formation of a film and the loss of fluid is negligible.

From the Charlotte Drake Cardeza Foundation, Division of Hematology, Department of Medicine, Jefferson Medical College and Hospital.

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CHEMICAL

LABORATORY TESTS OF THE OXIMETER WITH AUTOMATIC COMPENSATION FOR VASOMOTOR CHANGES

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THE two-filter oximeter as designed by Millikan[1] is an instrument for measuring the per cent saturation of the blood hemoglobin with oxygen. The apparatus consists of an earpiece, a small control box, and a galvanometer. The earpiece is clamped on the ear and the apparatus is standardized while the subject breathes pure oxygen. After standardization, the per cent saturation of the blood hemoglobin with oxygen can be read from the galvanometer scale at any time. For ease and convenience, this method has considerable advantage over the chemical analytical method since it requires no arterial punctures and gasometric analyses. Furthermore, the value of the oxygen saturation can be obtained instantaneously, without a wait of twenty or thirty minutes for the results of a chemical analysis. At the present time this method has found its main use in the altitude indoctrination program, but its use can be extended to experimental human physiology and to operating room procedures.

The earpiece of the oximeter contains a small lamp placed on one side of the ear and two photoelectric cells on the other. Special Wratten filters are placed in front of each photocell. One filter (called the green filter) transmits light which is absorbed by both reduced and oxygenated hemoglobin, while the other filter (the red filter) transmits light which is absorbed by reduced hemoglobin. Light passing through the ear passes through the respective filters and is received by the photocell. The photoelectric current from the photocell with the green filter is inversely proportional to the total amount of hemoglobin plus oxyhemoglobin (ear thickness), while the photoelectric current of the red-filter photocell is inversely proportional to the amount of reduced hemoglobin in the light path. In the older instruments five galvanometer scales were used, the choice of scale depending on the reading of the green filter photocell. When vasoconstriction or vasodilation occurred in the ear, it was sometimes necessary to change from one scale to another during an experiment. In a more recent modification, an improvement has been made in which a single scale is used and the circuit has been changed to allow automatic adjustment for vasomotor changes. The details of the revision are given by Millikan.[1] Since the changes in the circuit involved some approximations which would reduce accuracy, it was necessary to perform a series of calibration experiments.

*On leave of absence from the University of Minnesota.
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METHOD

The oximeter circuits used are shown in Fig. 1. The photocells of the earpiece are designated by *G* (green) and *R* (red). A small panel box contains the potentiometers and the dpdt switch. When Coleman earpieces (Coleman Electric Company, Maywood, Ill.) were used, a Leeds and Northrup type R galvanometer 2500b (CDRX 10,000 ohms and sensitivity 0.0005 microamperes per millimeter at a scale distance of 1 meter) was found to have more than the required sensitivity and performed satisfactorily (see oximeter circuit *A*). Another oximeter circuit *B* was identical with circuit *A* except that in place of the high-sensitivity galvanometer, a direct-current amplifier was used with an Esterline-Angus recording milliameter having a sensitivity of 1 milliampere full scale. With circuit *B* a written record of the changes in oxygen saturation was obtainable, whereas with circuit *A* it was necessary to take galvanometer readings visually. A record from the recording oximeter is shown in Fig. 2 which illustrates the changes in per cent oxygen saturation of the blood when a subject breathing 100 per cent oxygen is changed to 8 per cent oxygen plus 92 per cent nitrogen and then returned to 100 per cent oxygen.

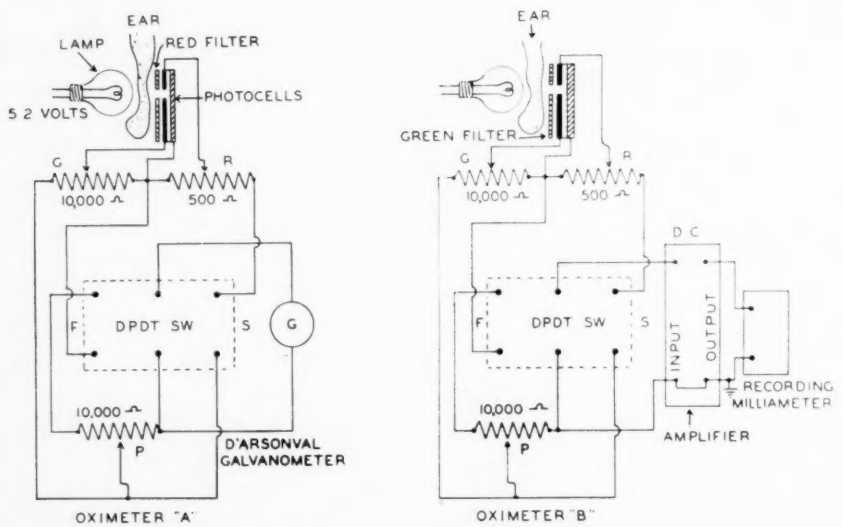

Fig. 1.—The two oximeter circuits used are identical except that the circuit labeled "galvanometer" has a d'Arsonval galvanometer to indicate blood oxygen saturation values and the circuit labeled "EA Recorder" uses a direct-current amplifier and an Esterline-Angus 1 milliameter recorder.

The electrical operation of this circuit is described in detail by Millikan.[1] The setting of the potentiometer *P* is determined by setting the galvanometer at zero in midscale and moving the sliding contact of the 500-ohm (red filter photocell) potentiometer *R* to zero. The dpdt switch is thrown to *S*, and *G* is adjusted until the galvanometer reads full scale. The switch is then thrown to the *F* position and *P* is adjusted until the galvanometer, now deflecting in the opposite direction, reads exactly $\frac{1}{5}$ of the reading in the *S* position. Once *P* is set it remains fixed for all readings.

The oximeter scale reads from 50 to 100 per cent on a logarithmic scale. In operation the galvanometer is adjusted to the scale zero (50 per cent on the

scale) and the "neutral filter" is placed in the earpiece. The apparatus is allowed to "warm up" for twenty minutes until thermal equilibrium is attained. The switch is thrown to *F* and *G* is adjusted until the galvanometer reads 100. The earpiece is then clamped on the subject's ear and allowed to remain for fifteen minutes until thermal equilibrium on the ear is attained. The subject then breathes 100 per cent oxygen, the switch is thrown to *S*, and potentiometer *R* is adjusted until the galvanometer again reads 100.

In testing the instruments, aviation students were used. They were fitted with oxygen masks and allowed to breathe pure oxygen while the preliminary standardization was being made. The breathing mixture was then changed to 8 per cent oxygen plus 92 per cent nitrogen. The oxygen content of the blood fell progressively as indicated by Fig. 2, and when the per cent saturation had reached 65 to 70 per cent, a sample of arterial blood was drawn under oil from the femoral artery. Oxygen content and oxygen capacity were determined by the Van Slyke manometric method and the per cent saturation computed. In a few experiments two samples of arterial blood were drawn, the first when the subject was breathing 100 per cent oxygen and the second when he was breathing 8 per cent oxygen.

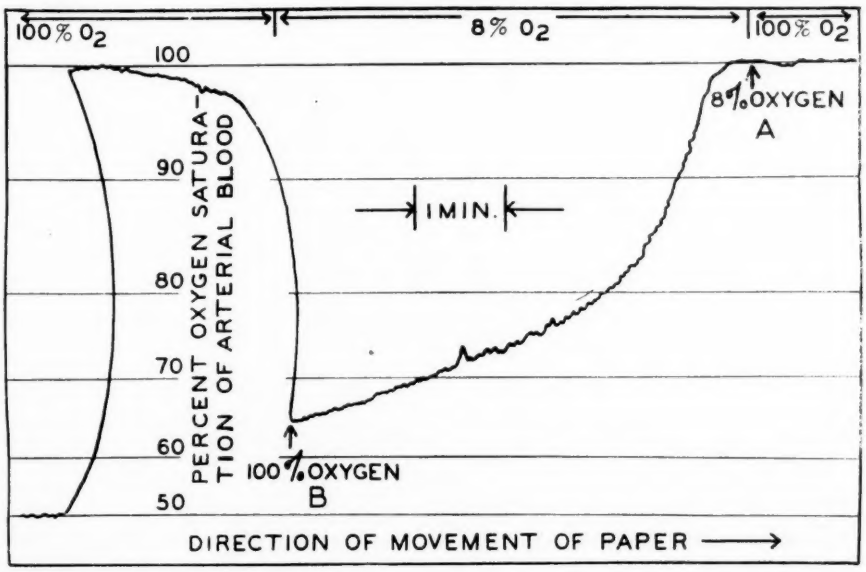

Fig. 2.—Record from recording oximeter. At the time indicated by *A*, the subject who was breathing 100 per cent oxygen was changed to 8 per cent oxygen plus 92 per cent nitrogen. At *B* the reverse change back to 100 per cent oxygen was made. In the interval *A-B*, the 8 per cent oxygen-92 per cent nitrogen was breathed.

RESULTS

The results are plotted graphically in Fig. 3. The electrical values read from the instruments are plotted on the abscissa, and the chemical values, obtained by computation from Van Slyke analyses, are plotted on the ordinates. Values obtained from oximeter *A* are plotted in Fig. 3 in the graph with "galvanometer" on the ordinate. Values for oximeter circuit *B* are in the graph with "EA recorder" in the ordinate. The line at 45° in the graph indicates

identical values. The two lines above and below indicate regions of ±2 per cent and ±5 per cent. It is to be noted that agreement within ±5 per cent is obtained for 89 per cent of the values using oximeter *A* and 83 per cent of the values using oximeter *B*. In all, 37 experimental tests were performed.

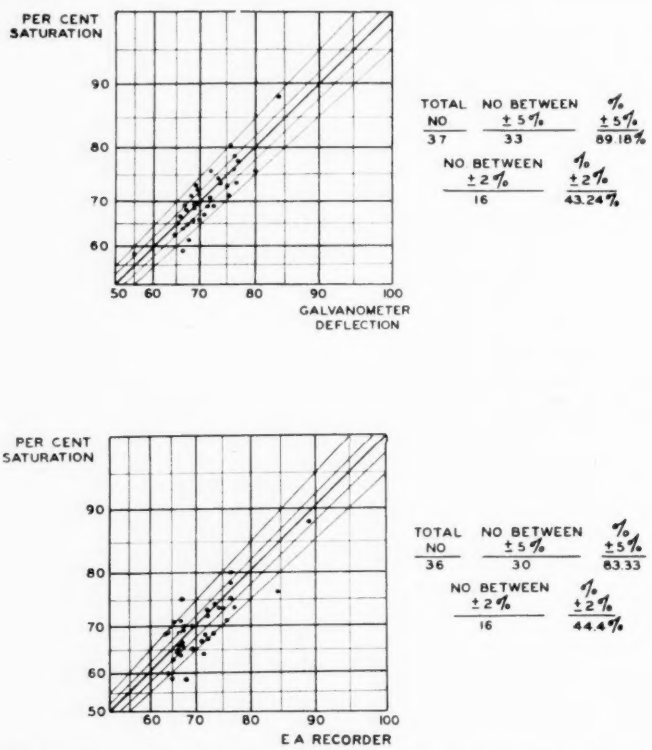

Fig. 3.—The galvanometer readings are plotted on the abscissa and the corresponding chemical oxygen analyses on the ordinate. For exact agreement, the points should fall on the heavy middle line sloping at 45°.

DISCUSSION

The agreement between the electrical and chemical values is approximately the same for both types of oximeter. It had been anticipated that the high-sensitivity galvanometer method (*A*) would give closer checks with chemical analyses than the amplifier method (*B*) due to the general unsteadiness with high amplification. The results show that this expectation was unwarranted and that the recording method gives values which agree well with the d'Arsonval galvanometer values.

It is believed that in this method of testing agreement of better than 5 per cent between electrical and chemical methods is not possible. There are certain random errors inherent in the method of testing which would account for a discrepancy of 2 to 5 per cent even if the oximeter gave the correct instantaneous values. These errors are the following:

a. *Errors in Chemical Analyses.*—The Van Slyke manometric method for blood oxygen is capable of giving duplicate values for oxygen content which agree within 0.5 per cent in the absolute value. The duplicates for oxygen

capacity are not as reliable but most of the values agree within 1.5 per cent. In terms of per cent saturation, the duplicates will have a maximum error of 2 per cent (in oxygen saturation) and usually check within 1 per cent. Also the Van Slyke method measures total blood oxygen, while the oximeter, recording the amount of reduced hemoglobin, measures only hemoglobin oxygen.

b. *Changing Oxygen Content of Blood.*—When subjects breathe 8 per cent oxygen plus 92 per cent nitrogen, there is a progressive fall in arterial oxygen content with the rate of fall varying from one individual to another. Superimposed on the more or less regular rate of fall of blood oxygen, there are fluctuations caused by deep and irregular breathing due to the carotid sinus stimulation. It requires thirty seconds to one minute to draw a sample of arterial blood, and in this time interval the arterial content varies in an amount equal to 1 to 5 per cent of the saturation value. The sample of blood in the syringe will represent an almost unpredictable average since it is drawn from the artery at rates which may vary.

c. *Error of Simultaneous Sampling.*—This error, which is probably small and is estimated to be less than 1 per cent, is the error involved in simultaneity of oximeter readings and blood sampling. Since the blood oxygen content is changing during the period in which blood is withdrawn, an exact record of the oximeter readings in this period is necessary.

When these three sources of error are considered, it is possible to have a discrepancy between analytical and oximeter oxygen saturation values in an amount as high as 5 per cent if the oximeter recorded the true oxygen value. These errors are inherent in the method of testing and are not errors due to faulty performance of the oximeter. The over-all average values differ by less than 1 per cent. For these reasons the error involved in using the oximeter is believed to be well within the value of 5 per cent which can be considered as a maximum.

SUMMARY

The Millikan type oximeter with automatic compensation for ear thickness has been tested by chemical analysis of arterial blood drawn from subjects breathing a mixture of 8 per cent oxygen and 92 per cent nitrogen. Agreement within ±5 per cent was obtained for values of oxygen saturation as low as 60 per cent. Two instruments were tested and found to give similar values. One instrument used a d'Arsonval galvanometer and the other a recording milliammeter with a direct current amplifier.

REFERENCE

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MODIFIED KOCH PIPETTE FOR CARR-PRICE REAGENT DISPENSER

D. B. Parrish, M.S., and M. J. Caldwell, M.S., Manhattan, Kan.

IN THE Carr-Price method of determining the Vitamin A content of blood[1] and other biological materials, difficulty is often experienced due to the reaction of the reagent with atmospheric moisture. If the antimony trichloride reagent is allowed to come in contact with the water vapor of the air, the inner surface of the delivery pipette soon becomes encrusted with the reaction product; hence special precautions must be observed in the use of the reagent.

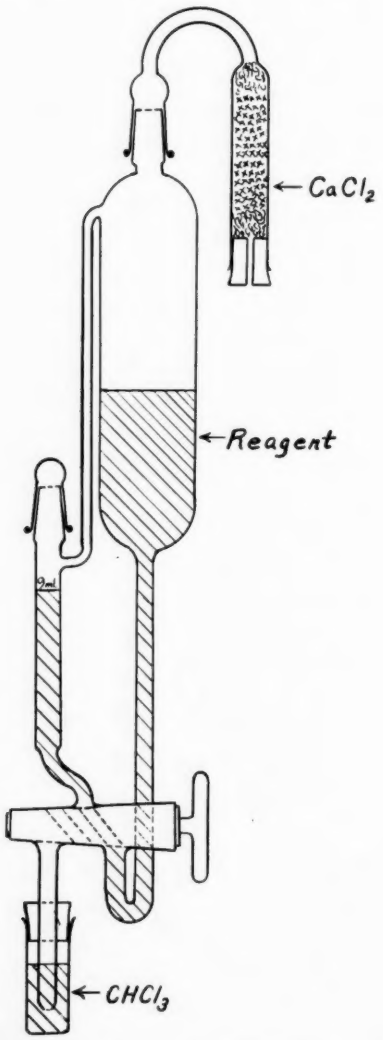

Fig. 1.—Modified Koch pipette for Carr-Price reagent dispenser.

From the Kansas Agricultural Experiment Station. Contribution No. 290, Department of Chemistry.
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Recent publications of Oser et al.[2] and Swain[3] have described special apparatus for dispensing the reagent. The difficulties encountered in handling the Carr-Price reagent have been largely eliminated in our laboratory by the use of the modified Koch pipette shown in Fig. 1.

The pipette is built around the three-way, large-bore (3 mm.) stopcock which will deliver 9 c.c. of reagent in approximately two seconds. The reservoir of about 200 c.c. capacity is connected to the under side of the stopcock by means of a 180° bent tube, which also serves as a settling chamber. The air breathing into and out of the pipette has been predried by passage through a calcium chloride tube and is in equilibrium with the antimony trichloride solution in the reservoir. The pipette will remain free of hydrolyzed products indefinitely. Fresh solutions of the reagent are introduced by funnel into the top of the reservoir, which is closed by means of a ground glass fitting, connected to a calcium chloride tube.

In use, the pipette is supported on a ring stand directly above the reaction cell in the photometer. The rapid delivery of 9.0 c.c. of reagent to 1.0 c.c. of the chloroform solution of vitamin A insures adequate mixing. After delivery of the reagent, a filter paper is touched to the delivery tip to remove the residual drop of solution. The tip is kept submerged in chloroform when the apparatus is not in use, and remains clean.

Since all parts of the apparatus which come in contact with the antimony trichloride solution are of glass, there is little chance of contamination. A good grade of stopcock grease used in moderation prevents leakage and has not been observed to interfere with the determination. The fact that the reagent may be allowed to remain in the apparatus from day to day results in considerable convenience and saving of time.

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2. Oser, B. L., Melnick, D., and Pader, M.: Estimation of Vitamin A in Food Products, Ind. Eng. Chem., Anal. Ed. **15**: 724, 1943.
3. Swain, Lyle A.: A Carr-Price Reagent Dispenser, Ind. Eng. Chem., Anal. Ed. **16**: 241, 1944.

MEDICAL ILLUSTRATION

PHOTOGRAPHY IN COLOR OF FIXED PATHOLOGIC SPECIMENS

MILTON G. BOHROD, M.D., AND JOHN J. BEITER, ROCHESTER, N. Y.

THE advantages of color photography in the recording of gross pathologic specimens are so obvious that they need no discussion. Only the high cost of reproduction prevents the complete replacement of black-and-white pictures in books and journals; the use of color transparancies is, however, becoming widely and successfully used in pathology laboratories.

Unfortunately, there are some situations in which the usual direct method of color photography is not available or gives unsatisfactory results. Mostly this occurs with small specimens: they may be received already fixed in formaldehyde; they may be brittle or friable; a few unavoidable high lights, not particularly harmful or even helpful in a large specimen, may obscure most of the pathology in a small one. Or it may be desired to photograph a cut section of the specimen, which in the unhardened state may fall apart on cutting or for other reasons may be difficult to cut.

It is our purpose to report a method which we have found to give excellent results in many cases where, for any reason, we have been unable to photograph the fresh specimen. The method is a combination of procedures which have been used many times before, even in black-and-white photography; this is one of its advantages, for it requires little equipment that is not already available to those who are taking photographs. Although it is designed principally for the small specimen we have found and will indicate some types of large specimens which can be treated by slight modifications of the usual method.

Essentially the method consists of bringing back the color of the fixed specimen by means of alcohol, just as in the preparation of a museum specimen, photography under alcohol, and the use of color backgrounds. In each of these steps, however, there are important "tricks" which spell the difference between good color and the washed-out, muddy appearance of many color photographs of fixed specimens.

FIXATION

We receive many of the small specimens we wish to photograph already in fixing solution, a procedure no pathologist desires but which is necessary at the present time in many hospitals. Even as little as 10 or 15 minutes of fixation will spoil such a specimen for immediate photography, and it is better to allow fixation to proceed than to try to rescue a specimen already in solution. Most of our specimens are fixed in 10 per cent formaldehyde, 1 part 40 per cent formalin plus 3 parts water. Since this gives excellent results we have done

From the Department of Photography of the Laboratories of the Rochester General Hospital, Rochester, New York.

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little work with other solutions, such as Kaiserling, especially since the solutions with a high salt content are undesirable for the subsequent microscopic study of the tissue.

The critical consideration at this stage is the *time* of fixation, which should be long enough for adequate penetration of the specimen but otherwise as short as possible. With larger specimens only the portion photographed need be fixed completely. For most of our small specimens, such as an appendix, a fallopian tube, small tumor, an eye, and similar organs, 24 hours is the optimum time; 12 hours may be enough. Beyond this time one may or may not get good results, and the time of fixation which may be used will vary inversely with the amount of blood in the specimen. Thus, an eye can be photographed well even after three or four days' fixation, whereas a hemorrhagic cyst of an ovary, for instance, may be ruined for good color photography after 36 to 48 hours of fixation.

Large specimens require longer periods for adequate fixation, otherwise only the outer layers in contact with the fixing fluid will be hardened. For this reason color photographs of large fixed specimens usually give poor color reproduction.

WASHING

After fixation the specimen is washed in water, running water if the specimen will stand it, otherwise in multiple changes of water. The period of washing need not be prolonged, a half to one hour being sufficient. It is during the washing process that the specimen is cut if it is desired to photograph a cut section.

RETURN OF COLOR

This stage should not be started until one is about ready to take the photograph. The specimen is put into 80 to 95 per cent ethyl alcohol and watched carefully. The original colors will begin to return in a few moments and will reach their peak in 10 to 30 minutes. If the specimen remains in alcohol much longer the colors will first lose their brightness, then begin to fade.

SPECIMEN TANK

Just before it is photographed the specimen is transferred to a small tank containing enough alcohol to cover it and is carefully placed in position in or near the center. Care must be taken to prevent particles of tissue or other foreign matter from floating in the fluid or settling on the bottom, for they will show in the photograph as defects in the background, especially if color backgrounds are used. For this reason, as well as for possible discoloration of the fluid by blood, we do not put the specimen directly into the tank after the stage of washing.

The glass of the tank must be free from bubbles, lines, rings, or other defects, for all of these appear in exaggerated form in the picture. Ordinary crystallizing dishes, the various sizes of which would suggest their use, are unsatisfactory. We have made a useful tank measuring 5 by 7 by 2½ inches by cutting 5 by 7 inch cover glasses and cementing the edges together with a thick solution of Clarite in xylol. The length of the tank should be almost twice the

length of the specimen. The sides must be low, so that the light reflected from the top and bottom edges of the sides and refracted through the solution will not form streaks in the field of the picture.

BACKGROUND

We have described our use of colored backgrounds in detail in another publication.[1] We are convinced that their use not only gives pleasing results but also aids in the visualization of the structures intended to be portrayed; but in no situation are the results more striking than in the photography of small specimens. Briefly, we use an illuminated box with the lights at the bottom; the specimen, in this case the tank containing the specimen, is placed on a plate glass above. About 6 inches below the upper glass plate a colored glass is inserted. The color of the background varies not only with the color of the glass and the strength of the lights beneath it, but with the intensity of the incident light on the specimen.[1]

PHOTOGRAPHIC EQUIPMENT

Any equipment which takes satisfactory color pictures with fresh specimens can be used in this method. A large aperture (f:4.5) lens is necessary for focusing when a scale of $\times 1$ or $\times 2$ is desired. Proper lamps, 3200° Kelvin, 500 W, in reflectors for Kodachrome type B film are indispensable for good color rendering. We use 3¼ by 4¼ inch film because we think the better results more than justify the additional expense over miniature film.

EXPOSURE

When the specimen is twice the diameter of the photo-electric cell of the usual exposure meter or larger, there is no difference between obtaining the proper exposure in this from ordinary color photography. If the specimen is small, the exposure can be obtained with reasonable accuracy by placing the palm under the plate glass beneath the specimen. In any case, while measuring exposure the lights of the illuminated box *must be turned off*.

A few specimens with which we have worked require slight modifications from the method mentioned.

EYES

Rarely does one desire to photograph the outside of an enucleated eye. External pathology is better photographed on the patient before the eye is removed. Cross sections are exceptionally difficult to make without dislocating the lens and otherwise disturbing anatomic relations; good sections are practically impossible on a fresh eye. The following method usually gives good results, but even with the utmost care one will sometimes displace or entirely dislodge the lens.

The eye is fixed in formalin for 24 to 48 hours and washed in water. Over a period of 24 hours it is then put in ascending strengths of alcohol, starting in 50 per cent and ending with 95 per cent. Then a single cut section is made, slightly above the level of the outer edge of the iris. The specimen is then carefully lowered into alcohol for several hours of additional hardening. The last section, parallel to the first, is then made.

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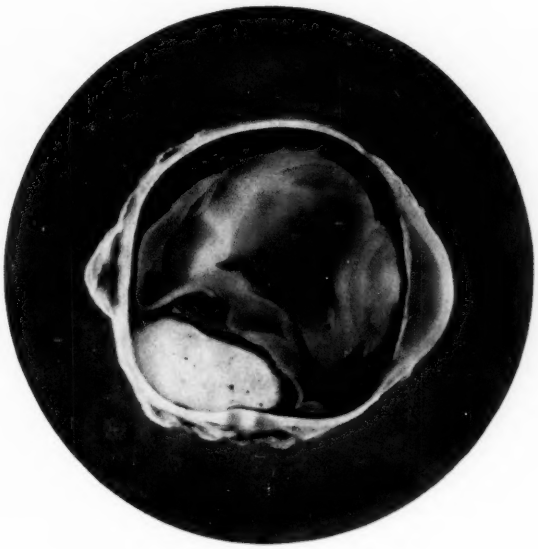

Fig. 1.—Leucoblastoma of the eye. Detachment of the retina. Fixed in formalin 4 days, then sectioned. Photographed in tank under alcohol.

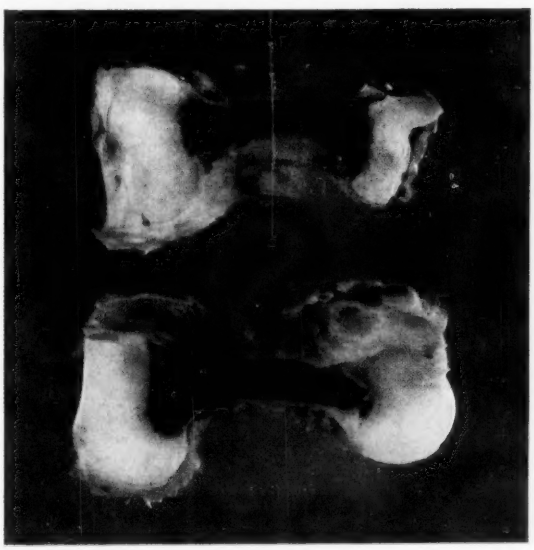

Fig. 2.—Achondroplasia. Transected femur and tibia. Fixed in formalin 24 hours. Photographed in tank under alcohol.

BRAINS

Many pathologists prefer to fix brains immediately upon removal. Certainly a fresh brain is not easy to handle and, if cut into, anatomic details may be obscured. Good results may be obtained if the brain is cut in four or five days, and the sections for photography are placed in alcohol. Since we have no tank large enough for a complete brain section, we either wash the specimen in water after it is removed from the alcohol and photograph it as we would a fresh specimen, or we keep it moist with alcohol, by the use of a dropping bottle, while it is on the plate of our box. The latter method is often to be preferred because some specimens become grayish after washing in water.

The successful photography in color of brains after fixation is probably the result of the small range of color in this organ. However, the delicate colors of meningeal purulent exudates are not too well preserved so that if possible such brains should be photographed while fresh.

OTHER ORGANS

External views of kidneys are fairly good after fixation. Sometimes the details of speckled lesions, such as in embolic nephritis, show up even better than in the fresh state, although the color of the renal tissue itself may not be exactly as it was when fresh. Sections of the spleen may be difficult to photograph fresh especially if, as in leucemia, they develop a film of coagulation on exposure to air. In such cases a section about 1 cm. thick may be fixed for 24 hours, a thin slice cut off the top and then treated as above. Fixed hearts give mediocre results. We have never obtained even a passably good picture of a fixed lung.

REFERENCE

1. Beiter, J. J., and Bohrod, M. G.: Transilluminated Color Backgrounds in Medical Photography, J. Biol. Phot. A. In press.

BOOK REVIEWS AND NOTICES

Hypertension and Hypertensive Disease. By William Goldring, M.D., Associate Professor of Medicine, New York University College of Medicine; Chief, Nephritis and Hypertension Clinic, New York University Clinic; Physician to the Adult Cardiac Clinic and Associate Visiting Physician, Third (New York University) Medical Division, Bellevue Hospital; Visiting Physician, Goldwater Memorial Hospital, and Herbert Chasis, M.D., Assistant Professor of Medicine, New York University College of Medicine; Associate Chief, Nephritis and Hypertension Clinic, New York University Clinic; Physician to the Adult Cardiac Clinic and Assistant Visiting Physician, Third (New York University) Medical Division, Bellevue Hospital; Cardiologist to the French Hospital. The Commonwealth Fund, 41 East 57th Street, New York 22, New York. Price $3.50. Cloth with 253 pages.

The purpose of this book is a "concise presentation of our (the authors') concepts of hypertension and hypertensive disease." It is recognized that "such a book must contain some dogmatism." The discussions of the possible identity of essential and experimental renal hypertension, of the uses of thiocyanate and nephrectomy, of the origin of hypertension in renal ischemia—to all of which they are opposed—vigorously sustain this purpose. The interest of these discussions is dulled by the insertion of beautifully printed tables—for instance, Tables 17 and 18—which abundantly and uselessly confirm points clearly stated in the text. The sections devoted to treatment are not completed by specific recommendations which might be of value to those less expert than the authors.

Throughout, the text is well written, and wherever it has held fast to the authors' intention, the presentation is excellent. When, to complete the argument, they find it desirable to stray from this purpose, concepts are introduced which, because of brevity, are necessarily incomplete, and therefore, sometimes misleading. The reader must carefully disentangle the serious opinions of the authors from the opinions they here attribute to others. The appendix contains descriptions of the measures they use for determination of cardiac output and renal function, and a discussion of the interpretation of renal functional data which should interest investigators in this field.

The student or clinician who would like to know the special point of view held by the authors will find it clearly presented in this book. Their experience and contributions have made that opinion well worth knowing, and worthy also of the dignified format selected by the publishers. The promise of a more rounded survey, implied in the title and suggested by the jacket blurb is not fulfilled. This, however, was not the authors' intention.

IRVINE H. PAGE, M.D.

Heart Diseases. By Paul Dudley White, M.D., Lecturer in Medicine, Harvard Medical School; Physician to the Massachusetts General Hospital, Boston. Third Edition, The Macmillan Company, 60 Fifth Avenue, New York. Price $9.00. Cloth with 1025 pages.

The new edition of this book maintains the high standards of the two previous editions. It is a skillful, comprehensive survey of diseases of the heart. Rapid advances in cardiology since the publication of the second edition in 1937 have necessitated a thorough revision. Significant alteration and augmentation have been made so that the book is larger than previously. A notable addition is the new first chapter on "The Range of the Normal Heart." Other important added sections include those on phonocardiography, recent knowledge concerning splanchnic resection for hypertension, a discussion of the surgical therapy of the patent ductus arteriosus, newer aspects of the treatment of subacute bacterial endocarditis, and an adequate and clear presentation of the present knowledge of precordial leads. Other new sections include intracardiac thromboses, pulmonary embolism, circulation of the blood and the cardiac aspects of military service, gastrointestinal diseases and miscellaneous conditions.

The book contains more illustrations than the previous edition. These are very well arranged. One of the most valuable features is the extensive and carefully chosen bibliography which follows every chapter. For a textbook covering a subject so full of details as cardiology, very little of importance has been omitted, although obsolete and unimportant material has been excluded.

A very worth-while chapter is the Addendum in which the author briefly presents information of current importance accumulated since the main body of the manuscript was sent to press. The volume is admirable for the clarity with which it is written and is highly recommended.

EDWARD MASSIE, M.D.

The Analysis and Interpretation of Symptoms. By Cyril M. MacBryde, Assistant Professor of Clinical Medicine, Washington University School of Medicine; Director, Metabolism Division, Barnes Hospital, St. Louis, Missouri, J. B. Lippincott Company, Philadelphia. Cloth with 301 pages.

In textbooks of medicine it is customary to present pictures of disease with combinations of symptoms which, taken together, enable the reader to recognize the conditions at the bedside. Mere recognition, however, does not necessarily imply understanding. It may be only through the analysis of each component manifestation that disease can be truly understood. Moreover, in the field of therapy mere recognition of a clinical entity is practically sufficient only when a specific remedy is available. In the absence of a specific remedy, treatment must depend upon the relief of individual symptoms, deep understanding of which is essential to effective management. For these reasons alone *Analysis and Interpretation of Symptoms* reprinted in book forms from Clinics, 1944, constitutes an important contribution. In the introduction the editor outlines admirably the purposes of the volume. He emphasizes that the analysis and interpretation of a symptom must depend primarily upon understanding of functional pathology, while its correlations with other clinical and labora-

tory manifestations and its relations to other diseases, although important, must be given secondary consideration. Nine subjects are discussed. It is appropriate that four of them relate to pain, the most common and troublesome of all clinical phenomena. The selection, as examples, of headache and of thoracic, abdominal, and joint pain permits wide scope in discussion. There is an excellent chapter on fever and a challenging discussion of nervousness and fatigue. Cough and hemoptysis are considered in their relation to various parts of the respiratory tract. Hematemesis and melena are competently considered both from the standpoint of their pathogenesis and their clinical relationships. There is a most stimulating chapter on obesity and a short but comprehensive discussion of jaundice.

Although in general the selection of topics has been fortunate, it is obvious that only a very few of the symptoms which are appropriate for similar treatment have been considered. It is to be hoped that these valuable and interesting discussions may be used as a nucleus for a more comprehensive book on the analysis and interpretation of symptoms.

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